

THE ROLE OF THE DOPAMINE SYSTEM

IN SOME MODELS OF

EXPERIMENTAL EPILEPSY

BY

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DEDICATION

This thesis is dedicated to  
MY WIFE and SON.



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## ABBREVIATIONS

cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
ARAS	ascending reticular activating system
A	anterior
L	lateral
V	vertical
BDH	British Drug House
cpm	counts per minute
dpm	desintegrations per minute
CO <sub>2</sub>	carbon dioxide
°C	degree centigrade
CT	chromatographically-tested
Ci	Curie
mCi	milli Curie
µci	micro Curie
nCi	nano Curie
CSF	cerebrospinal fluid
DA-B-oxidase	Dopamine-B-oxidase
DA	dopamine
NA	noradrenaline



5-HT	5-hydroxytryptamine
DC	decarboxylase
L-DOPA	L-3,4-dihydroxyphenylalanine
DOPA	3,4, dihydroxyphenylacetic acid
DHPG	3,4, dihydroxyphenylglycol
COMT	catechol-o-methyl transferase
MAO	monoamine oxidase
ECoG	electrocorticography
EDTA	ethylene diamine tetra acetate
GABA	gamma-aminobutyric acid
HCH	hexachlorocyclo hexane
HMPG	3-methoxy-4-hydroxy-phenylglycol
HMPG-SO <sub>4</sub>	3-methoxy-4-hydroxy-phenylglycol sulphate
HVA	homovanillic acid
5-HIAA	5-hydroxyindole acetic acid
HCl	hydrochloride
Hz	Hertz (cycles per sec)
[ <sup>3</sup> H]	tritium
i.p.	intraperitoneally
M	molar
mMol	millimole

$\mu\text{Mol}$	micromole
$\text{nMol}$	nanomole
$\text{pMol}$	picomole
$\text{ml}$	millilitre
$\mu\text{l}$	microlitre
$\text{g}$	gram
$\text{mg}$	milligram
$\mu\text{g}$	microgram
$\text{ng}$	nanogram
$\text{pg}$	picogram
$\text{NaOH}$	sodium hydroxide
$\text{nm}$	nanometer
$6\text{-OHDA}$	6-hydroxydopamine
$\text{OH}$	hydroxyl group
$\text{PTZ}$	pentylenetetrazol
$\text{S.D.}$	standard deviation
$\text{S.E.M.}$	standard error of the mean
$\text{TOH}$	tyrosine hydroxylase
$\mu\text{A}$	microampere
$\mu\text{V}$	microvolt
$\text{V}$	volt

Statements in terms of Ph.D. regulations 2.4.15 of  
the University of Edinburgh.

I hereby state that this thesis was totally composed by myself and all the work described was initiated by myself. All experiments were done by myself except:-

1. Dissection of brain regions for the tyrosine hydroxylase assay - which was done by Dr. D. Blackwood (MRC Brain Metabolism Unit, Dept. of Pharmacology).
2. The assay of dopamine and noradrenaline in striatum - which was done by Mrs. Y. Allison (MRC Brain Metabolism Unit, Dept. of Pharmacology).

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# ABSTRACT OF THESIS

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.....

The levels of 4-hydroxy-3-methoxyphenylglycol (HMPG) and homovanillic acid (HVA), the major metabolites of and reflecting the turnover of noradrenaline and dopamine, respectively, were estimated in the frontal cortex and striatum of cobalt-implanted rats. Previous reports showed dramatic reduction of tyrosine hydroxylase activity and compensatory reduction in catecholamine catabolising enzymes in cortical foci and striatum of this model. No significant change was found in the concentration of either metabolite in the brain regions studied. It was suggested that impairment of the saturable transport mechanism and possibly of the enzymatic conjugation of these metabolites prevented the expected reduction in their levels in those brain regions.

The normal concentration of noradrenaline in cortical foci, paralleled by similar findings in the striatum could have contributed to the neuronal hyperexcitability in this model. Post-mortem studies on brain tissue showed that HVA is more stable than HMPG under different conditions. These factors might have contributed to the higher HMPG levels previously reported in the cobalt model.

Elaborating on the role of the dopamine system in the cobalt model of epilepsy, it was found that lisuride, apomorphine, bromocryptine and CF 25-397, in that order of potency, suppressed epileptic activity in a dose-dependent manner. The chronic administration of bromocryptine attenuated the development of epileptic changes, particularly in the secondary focus. These effects were blocked by the dopamine antagonist pimozide, which itself potentiated the epileptic cortical changes. This finding contrasted to the lack of effect of the anti-cholinergic

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-2-

agent, hyoscine, on epileptic activity. Similar anti-epileptic effects of dopamine agonists were also seen in other forms of sensory epilepsy.

The intrastriatal administration of dopamine or apomorphine desynchronized the epileptic cortex, while destruction of catecholamine terminals in the striatum potentiated the epileptic changes. Moreover, the unilateral intrastriatal injection of dopamine inhibited epileptic activity in both cortical foci. It was concluded that dopamine agonists exerted their anti-epileptic effects, at least in part, by stimulation of the striatum.

The role of the dopamine system in subcortical foci induced by sensitization with low intensity stimulation (kindling) of the amygdala was also studied. Previous reports showed the inhibitory role of catecholamines in amygdaloid kindling. Tyrosine hydroxylase activity was found to be reduced in the stimulated, but not the contralateral amygdala, nor in the frontal cortex, hippocampus, hypothalamus, thalamus and striatum in kindled rats. This finding, together with the reduced dopamine content in the stimulated amygdala suggested that a reduction in the local dopaminergic neurotransmission was involved during the initiation of focal hyper-excitability changes in amygdaloid kindling. However, it seemed that spread of after discharge involved different mechanism. Dopamine agonists produced no significant effect on the rate of kindling development or on the local after discharge threshold and duration in kindled rats. These findings suggested that the striatum did not affect subcortical foci induced by kindling in the manner it influenced epileptic cortical foci following cobalt application. The lack of effect of dopamine agonists could further be explained by alteration in local receptor mechanisms in the amygdala. These mechanisms could also account for the observed present and past biochemical changes.

*Use other side if necessary.*

## SECTION I

SOME ASPECTS ON THE TURNOVER OF CATECHOLAMINES  
IN COBALT-INDUCED EPILEPSY IN THE RAT.

## INTRODUCTION

The individual experimental models of epilepsy have their separate advantages and disadvantages. In many of these models the epileptic behaviour lasts for a short time as in the case of epilepsy induced by the administration of systemic convulsants and epilepsies induced by the topical application of convulsant agents such as oestrogen or penicillin to brain tissue. The long term models are more relevant to clinical situations and include chronic epileptiform foci induced by freezing, the audiosensitive and photosensitive reflex epilepsies and the models induced by the topical application of heavy metals, particularly cobalt. The cobalt model is relatively cheap and simple to reproduce. Besides, various aspects of its biochemistry, histochemistry and pharmacology have been studied in the MRC Brain Metabolism Unit, Department of Pharmacology, Edinburgh University.

### A. THE COBALT MODEL OF EPILEPSY

#### 1. Review of literature

Kopeloff et al (1942) first reported that the application of powdered metal cobalt to the frontal cortex of monkeys produced epileptiform discharge in the electroencephalogram. Since that original observation, the application of cobalt as a powder or as a gelatin stick to the frontal cortex has been used to create reproducible epileptic foci in a variety of animals including the rat (Dow et al, 1962, Dawson & Holmes,

1966, Fischer et al, 1967). Dawson & Holmes (1966) observed that no motor seizures were seen if cobalt was implanted in cortical sites far from the frontal motor area of the rat brain. Cobalt powder has also been used to induce subcortical epileptic foci (Mancia & Lucioni, 1966).

Following the unilateral application of cobalt to the right frontal cortex, a primary epileptogenic focus develops in the tissue surrounding the implant. A distinct secondary focus is also formed in the contralateral cortex (Dow et al, 1962, 1972). Epileptiform spikes were maximal at 8 to 12 days and were less marked at 4 weeks after cobalt implantation. The behavioural response took the form of contralateral fore-limb clonic movements and bilateral whisker twitches occurring at 4 to 10 days following implantation (Dow et al, 1962). Furthermore, the animals developed a lowered threshold to systemic anti-convulsants within one week of the application of cobalt (Hartman, Colosanti & Craig, 1964). Ashcroft et al, (1974) suggested that the secondary focus arises as a response to the spread of epileptic activity from the primary focus across the corpus collosum, and possibly other pathways.

Histologically, the focal application of cobalt produced intense cellular damage with necrosis, together with focal meningeal inflammation (Payan, 1971, Fischer et al, 1968, Emson & Joseph, 1975). Degenerative changes were also seen in the secondary focus, as well as striatum and the thalamus on both



sides (Ashcroft et al, 1974). Cobalt ion was also detected in the secondary focus as well as the striatum and occipital cortex on both sides of the brain, as early as 6 days and also at 3 weeks following cobalt application to the right frontal cortex (Clayton & Emson, 1976) indicating axonal transport of cobalt ion (Pitman et al, 1972).

Dow et al (1962) suggested that the focal hypersynchronous epileptic activity characteristic of this model closely approximated epileptic EEG pattern of some human epilepsies. This was supported by Dawson & Holmes (1966). Ashcroft et al (1974) have found that ethosuximide, an anti-convulsant effective in petit mal, reduced epileptic activity in cobalt implanted rats while phenobarbitone and diphenylhydantoin were without effect. The pattern of response to anti-convulsants and the fact that cobalt rats showed focal motor epileptic signs but only rarely developed generalised convulsions suggest the cobalt model is more suitable for focal or myoclonic seizure or petit mal. From the biochemical aspect, this may be advantageous since biochemical analysis will not be overshadowed by the effects of generalised convulsions.

## 2. Biochemical alterations in cobalt-induced epilepsy

Emson & Joseph (1975) showed that the levels of glutamic acid decarboxylase and choline acetylase, enzymes that are respectively associated with gabbaminergic and cholinergic

systems, were reduced in the primary cortical focus induced by cobalt implant to the right frontal cortex. This reduction was maximal when epileptic spiking was at its peak, and the enzyme levels recovered rapidly as spiking declined. The concentration of several transmitter amino acids including gamma-aminobutyric acid and aspartate were also diminished in the primary focus as the spike frequency increased and they recovered as the spike activity decreased.

These changes in the primary focus were not consistently paralleled in the secondary focus. The levels of choline acetylase and glutamic acid decarboxylase were only slightly reduced while the concentrations of gamma-aminobutyric acid was slightly elevated in comparison to control value. This might mean that the changes in the primary focus are more due to the trauma of the implantation procedure itself than to a concomitant epileptic response. It might also indicate that the cobalt model represents a denervation response in the production of focal epilepsy (Emson & Joseph, 1975).

The activity of tyrosine hydroxylase was dramatically reduced in both the primary and secondary focus. This reduction was maximal when the spike activity was at its peak 8 days following cobalt implantation and started to recover by 4 weeks and completely returned to normal activity 10 weeks after the application of cobalt (Clayton & Emson, 1976). However, the concentration of noradrenaline in both foci did not differ using either biochemical methods of estimation

(Clayton 1976) or fluorescence (Emson & Bjorklund, unpublished data).

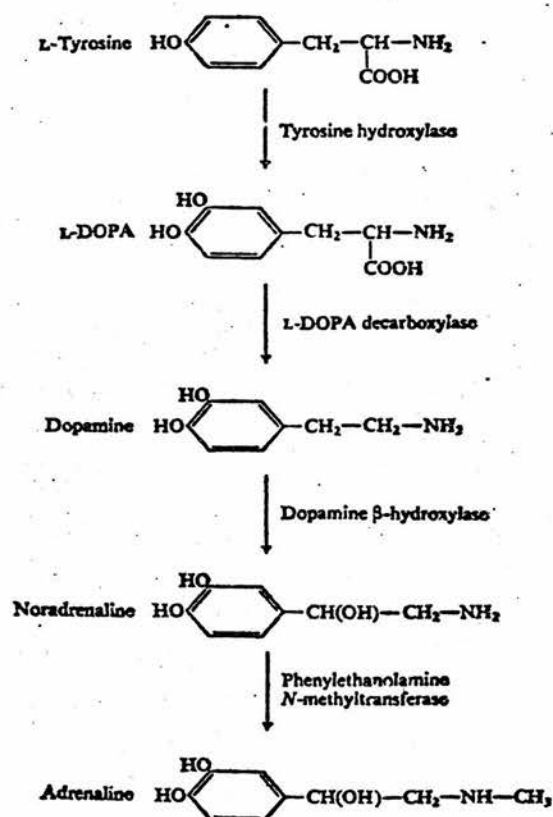
Morrel (1964) using histochemical methods, reported that the ribonucleic acid content was raised in epileptogenic tissues in cobalt-implanted cats. However, employing labelled precursors, Dewar et al (1972) showed no change in ribonucleic acid levels in epileptic foci and the occipital control area of the same rat brain.

## B. CATECHOLAMINE TURNOVER IN THE BRAIN

Each of the biogenic amines has its own peculiar distribution in the brain. It has been suggested that they serve as synaptic transmitters or modulators of normal activity in functionally distinct systems of the brain, namely 5-hydroxytryptamine (5-HT) in limbic structures, dopamine in the extra pyramidal and limbic system, and noradrenaline in central autonomic and alerting systems.

### 1. Synthesis (See Fig. 1)

The precursor amino acid tyrosine, crosses the blood brain barrier to become hydroxylated by the enzyme tyrosine hydroxylase at localized intracellular sites to form L-3,4, dihydroxyphenyl-alanine (L-DOPA). L-DOPA is then decarboxylated by the enzyme aromatic amino acid decarboxylase to yield dopamine. Dopamine is then taken up into storage organelles or vesicles, which appear to be coupled enzymatically or spatially, with ring



**FIG.1** Figure showing the major biosynthetic pathway of dopamine and noradrenaline.

hydroxylases (Moir & Eccleston, 1968, Udenfriend, 1968). The hydroxylation of dopamine inside these organelles or synaptic vesicles, leads to the formation of noradrenaline. It seems now that noradrenaline could be synthesized either in nerve cell bodies (Carlsson, 1966, Dahlstrom & Fuxe, 1964) or nerve terminals (Levitt et al, 1965). Vesicles, synthesized in the cell body, were transported down the axon to nerve terminals (Dahlstrom & Fuxe, 1964), whereby they would supplement the vesicle stores replacing the ones lost by synaptic transmission. Adrenaline, which is present in very small amounts in the brain, is formed by N-methylation of noradrenaline.

## 2. Catabolism

The mechanisms available for inactivation of catecholamines released from nerve terminals are active re-uptake and enzymic degradation by methylation, catalyzed by catechol-O-methyl transferase, and oxidative deamination catalyzed by monoamine oxidase.

### (a) Monoamine oxidase

This enzyme was found to be active in oxidative deamination of catecholamines (Blaschko et al, 1937). It is, however, not specific for catecholamines and has a wide distribution in most animal tissues. It is mitochondrial-bound (Hawkins, 1952) and consists of a flavoprotein containing 2 moles of FAD per mole of enzyme (Yasunobu, 1968). Cytochrome C1 and copper are co-factors.

Since it is bound to mitochondria, it has an intracellular location, and is found in nerve cell bodies and nerve terminals. The enzyme is more active with phenylethylamine and catecholamine derivatives lacking a  $\beta$ -hydroxyl group such as tyramine and dopamine than with noradrenaline and adrenaline (Blaschko et al, 1937, Blaschko, 1952) and is stereospecific for the L-form of the amines (Prakesi & Blaschko, 1959).

(b) Catechol-O-methyl transferase

This enzyme causes the transfer of a methyl group from S-adenosyl methionine to the 3-hydroxyl phenyl group of catechol (Axelrod, 1957). This enzyme is found in most cells and is very abundant in liver and kidney (Axelrod et al, 1959), and has been demonstrated in the brain, where it is thought to be a soluble enzyme, but may be membrane bound since it is difficult to purify free from membrane with enzymic activity.

Little is known about the enzymes which convert the aldehydes, formed by oxidative deamination of the amines by monoamine oxidase, to either the acid or the neutral metabolites. Since the intermediate aldehyde metabolites have not been demonstrated, the enzymes causing the further reduction or oxidation are probably intimately coupled with monoamine oxidase, and hence probably bound to mitochondria. Breese et al (1969), however, showed that the presence of a  $\beta$ -hydroxyl group will facilitate the formation of the neutral metabolites, whereas its lack will

result in acid metabolite although no real reason is put forward for this phenomenon.

The metabolic fate of catecholamines in brain, in vivo, had been shown following the fate of the labelled amine injected into the lateral ventricle of the rat brain (Mannarino et al, 1963). Three main groups of metabolites were demonstrated (Fig. 2):-

1. The 4-hydroxy-3-methoxyphenyl metabolites.
2. The 3,4, dihydroxyphenyl metabolites.
3. The sulphate or glucuronide conjugate of either.

The main metabolites of dopamine in brain tissue and CSF are 3-methoxy-4-hydroxy-mandetic acid (Homovanillic acid, HVA), (Sharman, 1960, 1963, Anden, Roos & Werdinius, 1963a and b), and 3,4,dihydroxyphenylacetic acid (DOPAC) (Rosengren, 1960, Ashcroft et al, 1968). DOPAC is formed by deamination of dopamine by monoamine oxidase (Fig. 2), and about one half of it is further O-methylated to form HVA. There is a large variation between animal species as the ratio between the concentrations of DOPAC to HVA differ considerably. In the mouse, the ratio is about one (Roffler-Tarlov et al, 1971), while in the rabbit (Anden et al, 1963) and in the dog (Moir & Yates, 1972), there is about ten times as much HVA as DOPAC.

It has been shown that the major metabolite of noradrenaline

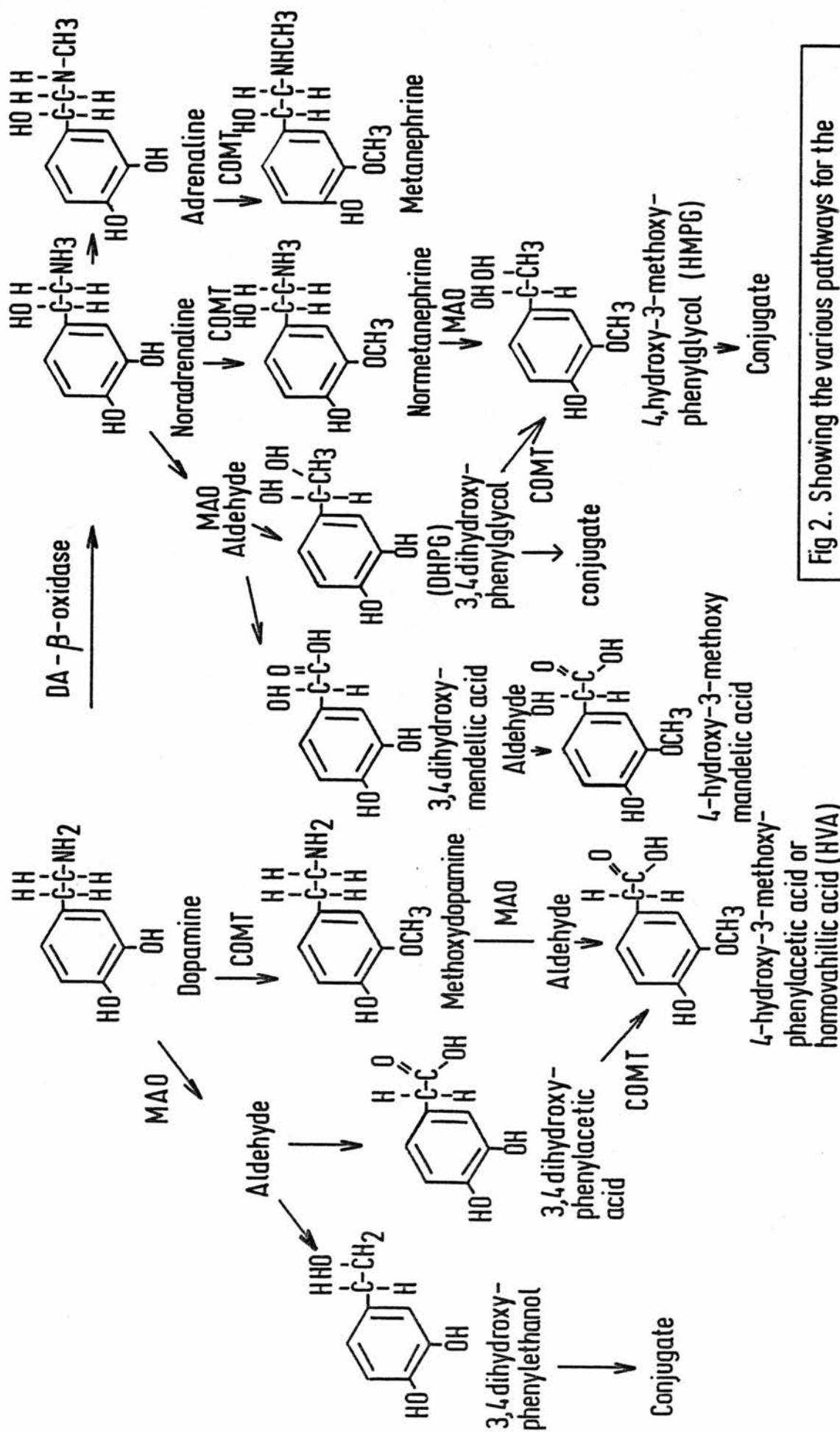


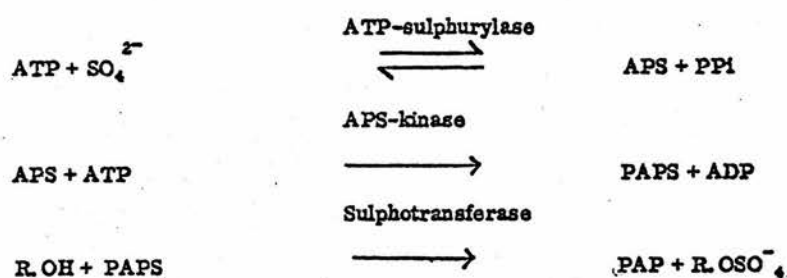
Fig 2. Showing the various pathways for the catabolism of dopamine and noradrenaline



in rat and cat brains by analysis of the radioactivity after intraventricular injection of the labelled amine, was 4-hydroxy-3-methoxyphenylglycol (HMPG) or its sulphate conjugate (Mannarino et al, 1963, Schanberg et al, 1968a and b, Breese et al, 1969). Moreover, Schanberg et al (1968b) showed that the major metabolite of both labelled noradrenaline and normetanephrine injected into the lateral ventricle of the rat was conjugated HMPG. The radioactivity in the combined free HMPG and vanillin mandelic acid portion was only ten per cent that of the conjugated fraction. Furthermore, they showed that the conjugate was probably a sulphate ester since hydrolysis occurred with sulphatase preparation, whilst  $\beta$ -glucuronidase preparation was ineffective. Moreover, HMPG was demonstrated as the major metabolite of noradrenaline in many other species including man (Schanberg et al, 1969, Sugden & Eccleston, 1971, Gordon et al, 1973).

The sulphate esters of noradrenaline metabolites are probably formed using intracellular-located enzyme system described by Roy (1971) where a two-stage process is thought to be involved (Fig. 3). The first stage is the activation of sulphate, itself a two-step process involving the addition of a sulphate group to adenosine monophosphate and the phosphorylation of the ribose 3-OH group using ATP. The second stage is the transfer of the sulphate group to the recipient phenol group.

The main part of the deaminated metabolites from dopamine



APS = adenosine 5<sup>1</sup>-sulphatophosphate  
 PAPS = 3<sup>1</sup> phosphoadenylylsulphate  
 PAP = 3<sup>1</sup> phosphoadenosine-5<sup>1</sup>phosphate  
 PPi = pyrophosphate

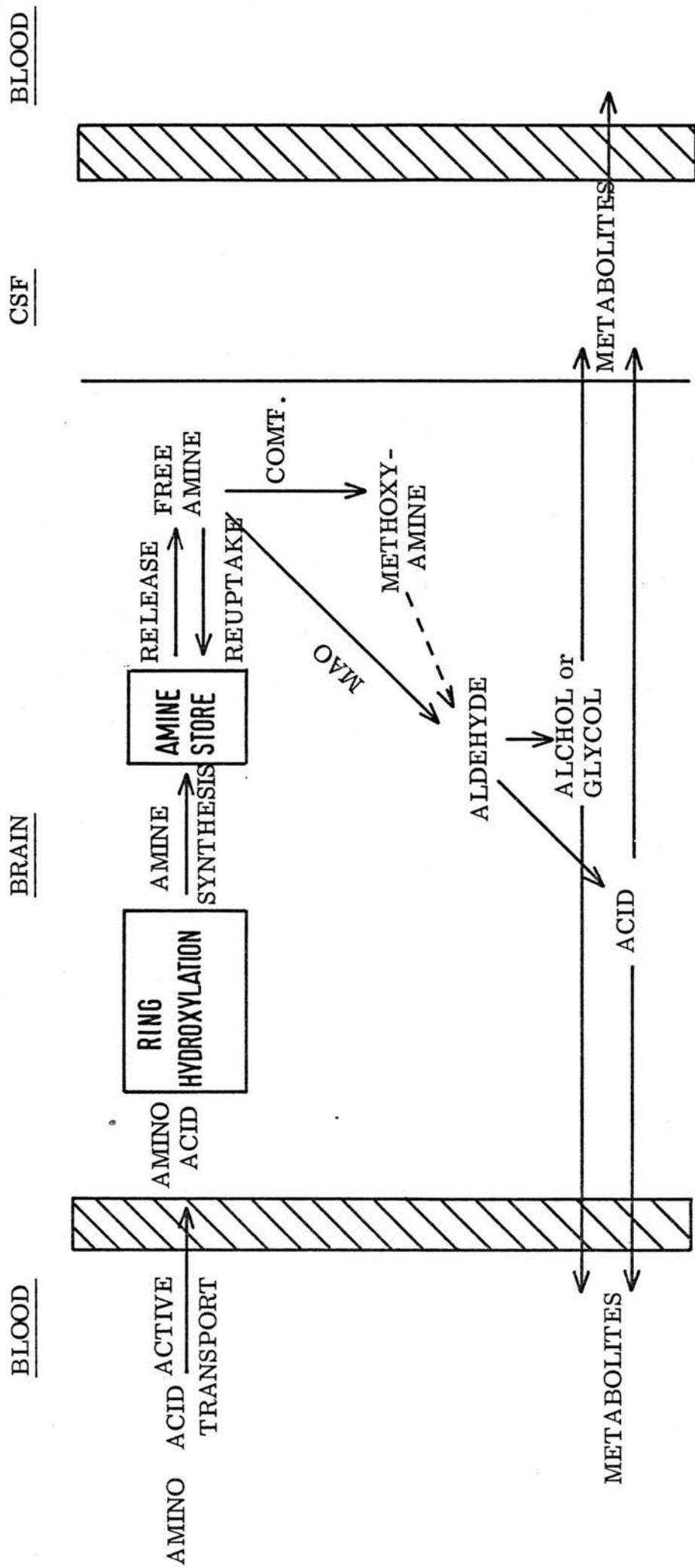
**FIG. 3** Figure showing the various stages of sulphation of phenolic compounds in the liver. Similar mechanisms are thought to occur in brain.

(Roffler-Tarlov et al, 1971) and from noradrenaline (Braestrup & Nielsen, 1975) are produced intraneuronally in the brain while the O-methylated/deaminated metabolite is formed mostly outside the nerve terminals.

Thus, dopamine is first deaminated inside the neuron into DOPAC and this is later O-methylated extraneuronally into HVA. However, a small part of HVA can be formed inside the neuron. Dopamine, released into the synaptic cleft, may first be O-methylated to form methoxydopamine (3-methoxytyramine) which is immediately deaminated inside the neuron into HVA. Moreover, both steps may take place intraneuronally as the enzyme catechol-O-methyl transferase has been shown to be also associated with nerve terminals (Broch & Fonnum, 1972). Similarly, noradrenaline is first O-methylated by catechol-O-methyl transferase extraneuronally to form normetanephrine which diffuses immediately inside the neuron to be deaminated by monoamine oxidase to form HMPG. A small amount of HMPG might be formed by O-methylation of 3,4-dihydroxyphenylglycol (DHPG), formed by deamination of noradrenaline inside the noradrenergic neuron.

HMPG, thus formed inside the neuron in the brain, is free and can diffuse freely outside the neuron into the blood or CSF. However, in the rat brain, most of this free HMPG is conjugated intraneuronally to form HMPG-sulphate. These major metabolites of dopamine and noradrenaline so formed must then cross barriers to either CSF or blood (Werdinius, 1967, Meek & Neff, 1972) (Fig. 4).

Fig 4



Schematic illustration of the metabolism of cerebral biogenic amines.

### 3. Methods of studying catecholamine turnover

Attempts were made to measure noradrenaline turnover in the brain as a possible clue to its functional importance. Since both catecholamines were restricted from entry into the brain by blood brain barrier (Weil-Malherbe et al, 1959), other ways of measuring turnover were described:-

1. Measurement of the rate of disappearance of endogenous noradrenaline after synthetic blockade using tyrosine hydroxylase inhibitor such as alpha-methyltyrosine.
2. The rate of disappearance of  $^3\text{H}$ -noradrenaline or  $^3\text{H}$ -dopamine injected into the lateral ventricle.
3. Measurement of the rate of disappearance of labelled noradrenaline or dopamine synthesized from labelled precursor, tyrosine, injected intravenously.

Using these methods, it was found that the turnover rate of catecholamines, as with their endogenous concentrations, was different in various brain regions, being highest in areas of low concentration of each amine and vice versa (Udenfriend, 1963, Burach & Draskoszy, 1964, Iversen & Glowinski, 1966).

### 4. Metabolite estimation

Another way of estimation of catecholamine turnover in the brain would be to look at changes in the major metabolite

level. These changes are measured either by:-

- A. A change in the specific activity of the main metabolite following labelling of the endogenous amine stores,  
  
and
- B. Assuming that the main metabolite itself cannot enter the brain from the blood, then the concentration of these metabolites in brain regions might be expected to provide an index of turnover of the parent amine.

However, two criteria must be satisfied:-

- (a) Defining the major metabolites of dopamine and noradrenaline in the brain, and this has already been shown to be HVA and HMPG, respectively.
- (b) Determination whether changes in the level of these metabolites reflect the turnover of the corresponding amine.

HVA and HMPG, which are the main metabolites of dopamine and noradrenaline, respectively, will not enter the brain readily from the circulation (Werdinius, 1967, Chase et al, 1973).

Intravenously administered HVA did not enter the brain or CSF of the rabbit (Bartholini et al, 1966) or CSF of the dog (Guldborg & Yates, 1968). Moreover, intravenously administered  $^{14}\text{C}$ -HMPG

does not readily enter the CSF of man (Chase et al, 1973).

HVA and HMPG sulphate are eliminated from the brain by enzyme-mediated saturable transport mechanism (Werdinius, 1967., Meek & Neff, 1972). Thus, these metabolites pass into the CSF and reach equilibrium with those of brain tissue. In normal tissue, blocking the transport system of these metabolites by probenecid would impair the passage of the metabolites from the brain to the circulation leading to their accumulation in the brain of the rat and cat (Werdinius, 1967, Sharman, 1967, Meek & Neff, 1972) and CSF of rat and dog (Guldborg et al, 1966, Meek & Neff, 1972, Ashcroft et al, 1975). In man, however, probenecid will lead to increase in CSF levels of HVA but not HMPG (Gordon et al, 1973, Korf et al, 1971, Chase et al, 1973). This probably is because HMPG in human brain is only 20% conjugated in contrast to 90% and 80% in the rat and dog brain, respectively (Schanberg et al, 1968b).

Local stimulation of the locus coeruleus, where nor-adrenaline-containing neurons originate (Ungerstedt, 1971) produced a significant increase of 60% in total HMPG in pooled right and left cerebral cortex (Walter & Eccleston, 1974), indicating the increased turnover of noradrenaline in the stimulated rats.

It was also shown that the rate of HVA formation in the brain reflected the rate of dopamine formation and turnover in the brain (Sharman, 1966, 1967).

Moreover, estimation of HVA levels in different regions of the brain was used in attempting to localize central dopaminergic fibers and is quite a reliable method since HVA is relatively stable in post-mortem tissue (Hornykiewicz et al, 1968).

### C. AIM OF THE WORK

Previous workers observed that the concentrations of 3-methoxy-4-hydroxyphenylglycol (HMPG) and homovanillic acid (HVA), the main metabolites of the catecholamine noradrenaline and dopamine, respectively, were altered in brain tissue taken from rats with cobalt-induced epilepsy. It is well known that alteration in the levels of these metabolites reflect changes in the turnover of their respective catecholamine precursor. Increased levels of HMPG were found in the epileptic cortical foci and striatum 8 days after cobalt implantation reaching 5-6 folds of the control levels, in contrast to HVA levels which showed a marked reduction in the striatum at that period (McQueen & Emson, unpublished results). This is in comparison to the high levels of HMPG and HVA in frontal cortex and striatum respectively, reported by Clayton (1973) after cobalt implantation, together with the reduced tyrosine hydroxylase activity in these structures.

The present work was done to confirm and possibly extend the findings in these metabolite changes, reflecting the turnover of their parent catecholamine.

These metabolites were first estimated 8 days following cobalt



implantation when epileptic activity was well established and any change in catecholamine turnover might be anticipated. Furthermore, post-mortem alteration of both metabolite levels were measured to determine the extent to which it might have contributed to the high levels already observed. It is hoped that this work could help in the extension of the role of catecholamine turnover in the focal epileptic changes induced by cobalt implantation.

## METHODS

### 1. ANIMALS

Male Piebald Virol Glaxo (PVG) rats, 200-250 g in weight, were used.

### 2. PREPARATION OF COBALT-GELATIN STICKS FOR IMPLANTATION

This was prepared according to the method of Fischer et al (1967). Metallic cobalt powder (Mesh 200) was used. To 5 ml of cobalt powder, 7 ml of 5% aqueous gelatin solution, warmed to 56°C was added, mixed and stirred at 56°C in a beaker.

After sedimentation of the suspended powder and decantation of the supernatant gelatin solution, the mixture was poured on a horizontal slide to form a layer about 0.75 mm thick. After cooling, the solidified cobalt-gelatin film (still on the slide) was dehydrated in acetone for 2 hrs, then kept in formaldehyde for 2 hrs for fixation. It was then washed with distilled water and kept in 80% alcohol.

Before use, the alcohol was allowed to evaporate. On the slide, sticks sized 0.75 x 0.75 x 1 mm were cut from the film using a sterile blade.

### 3. SURGICAL PROCEDURES

# A. Cobalt implantation into the right frontal cortex

The following procedure concerning the operation and subsequent electrocorticographic (ECoG) recording from cobalt implanted rats were all carried out in the MRC Brain Metabolism Unit, Edinburgh University, using the method of Dow et al (1962, 1972).

All surgical procedures were done under aseptic conditions. Male PVG rats (250-300 g) were anaesthetized with halothane (Fluothane, ICI) in oxygen (2.5 lt/min). A halothane concentration of 4% was used for induction and 2% for maintenance of anaesthesia. The scalp of the animal was shaved with an electric hair trimmer and then it was swabbed with 2% iodine solution in ethanol. A straight midline cranial incision (2-2.5 cm long) was made on the scalp and the underlying fascia was reflected to expose the outer surface of the skull. After locating the coronal and sagittal sutures, the skull was trephined, the hole was placed 2 mm from each suture in the right anterior quadrant. The hole was made with a dental drill (Renta model RA/21) and a round No. 6 burr (Ash), care being taken not to damage the underlying dura mater. Haemorrhage from the skull could be easily controlled and did not constitute a problem. The cobalt implant was made into the frontal cortex as follows:-

At the site of the implant, the dura was split with the tip of sterile 23-gauge needle. Using fine forceps, one mm cubes of the cobalt-gelatin stick was inserted perpendicularly

into the cortex so that its tip was flush with the cortical surface. The hole in the skull was then sealed with bone wax and the whole operative field was sprayed with antibiotic (Polybacterin). The incision was then closed with Michel clips (Aescular 12 x 3 mm) or silk (Mersilk). The whole procedure took about 15 mins.

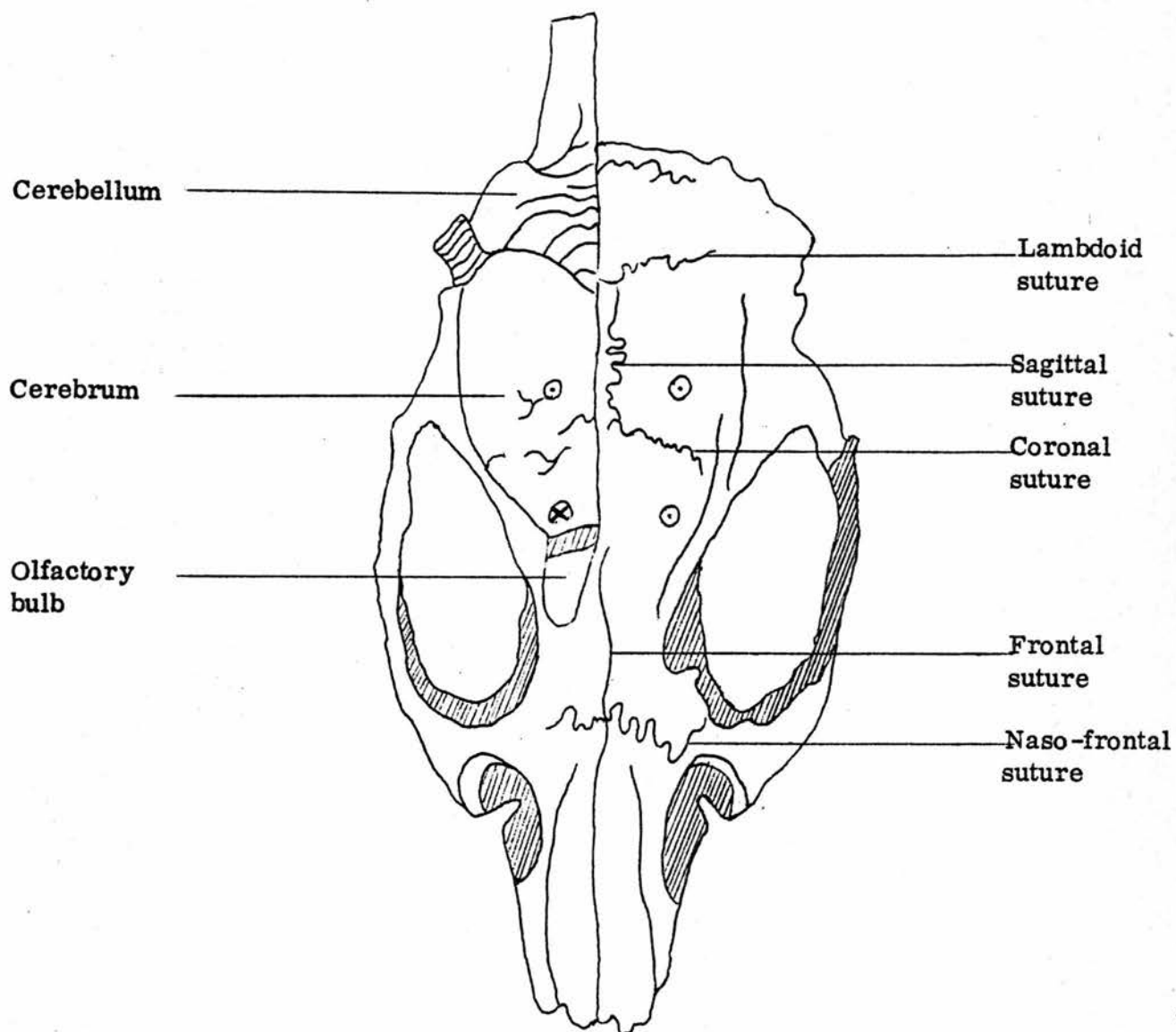
Control animals refer to both unoperated litter mates of the experimental animals and to sham operated animals. The latter were operated upon exactly as the experimental animals, but no cobalt was placed into the cortex. The types of controls used in each experiment is stated in the methods and the results.

#### B. Fixation of recording screws to skull

In cases of an animal whose EcoG was to be recorded, the operation procedure was modified so as to include the fitting of permanent recording electrodes. Using the same equipment and preoperative procedure as above, a curved cranial incision (2-2.5 cm) was made through the scalp close to the left eye. The skull was cleaned and the coronal, sagittal and naso frontal suture located and used as landmarks to ensure consistent placement of the recording electrodes. The skull was trephined, pairs of holes were centered 2 mm on each side of the sagittal and coronal sutures. Cobalt gelatin implantation was carried out into the right frontal cortex as described above. (See Fig. 5A).

Specially constructed stainless steel screws (8BA, about 8 mm in height, made in the Department) were inserted into the holes to act as recording extradural electrodes.

Fig. 5A



- ⊙ Electrode
- × Site of cobalt implant

Rat skull - dorsal aspect

One half of brain surface exposed

x3

The screws were secured to the skull by cold-curing acrylic resin (Simplex), and to aid this purpose, a groove had been cut around the body of the screw.

One of the screws was placed directly over the implant and one in each of the other three holes. Fig. 5B shows the design of the screws and their placement in the skull. The operation area was then sprayed with Polybactrin and the skin flaps replaced after four holes had been punched in it so that it fitted over the electrodes and allowed them to protrude externally for recording. The wound was then closed with Michel clips or silk. The whole procedure took 20-25 mins.

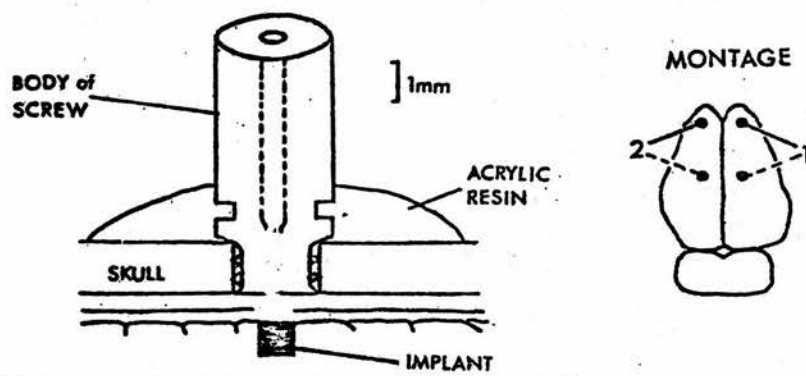
#### C. Post-operative management

No special post-operative care was necessary. Skin wounds healed quickly, and Michel clips removed after 7 days of operation. Drug-treated rats in a particular experiment were always kept separate from controls. Not more than 4 rats were placed in a cage, food and water were allowed ad libitum.

Rats were checked daily for the early epileptic manifestations. Few rats might have loose screws in the early post-operative period. These were fixed again with acrylic under aseptic conditions in the operating theatre. Older rats with loose screws were discarded from the experiment.

#### 4. ELECTROCORTICOGRAPHIC (ECoG) RECORDINGS

See Section II, Methods.



**FIG 5B** Placement of the hollow stainless steel screw in the skull.

## 5. COLLECTION AND STORAGE OF BRAIN SAMPLES FOR BIOCHEMICAL ASSAY

Groups of experimental rats were stunned, and decapitated between 3 and 11 days after cobalt implantation. The brains were removed quickly, placed on a mixture of solid CO<sub>2</sub> and crushed ice. As dissection was completed, the tissue samples, placed in polythene bags, were stored in liquid nitrogen (-195°C). No tissue sample was left in liquid nitrogen for longer than 3 weeks before being assayed.

## 6. DISSECTION OF BRAIN TISSUE SAMPLES

The brain was placed on its ventral surface on a glass plate resting on a mixture of solid CO<sub>2</sub> and crushed ice. The cerebellum was removed, and the forebrain bisected. The curved tip of a micro-spatula was introduced into the lateral ventricle and passed caudally and rostrally, reflecting the cerebral cortex laterally.

Tissue portions were taken from the following brain regions, using fine forceps:-

### (a) Right frontal cortex (RFC)

This contained the site of the implant. All visible pieces of cobalt gelatin as well as any calcified tissue were removed, before the sample was taken.



(b) Left frontal cortex (LFC)

This area is the site of the secondary (mirror) focus in cobalt implanted rats. No gross changes could be seen. From each frontal cortex, tissue samples were taken quickly and frozen on solid CO<sub>2</sub> before storage.

(c) Occipital cortex (OcC)

Larger tissue blocks were taken from this area, since it was used to estimate the tissue recovery of HMPG. This area was reported to show no biochemical or ECoG changes after cobalt implantation and so can be used as internal control for the same animal.

(d) Right striatum (RS)

(e) Left striatum (LS)

The striatum was identified as bilateral masses of gray matter in the floor of the lateral ventricle with the expanded head of the caudate nucleus anteriorly and its body and tail tapering posteriorly. Care was taken during dissection of the striatum to avoid including any portion of the adjacent cortex or white mater.

The average dissection time for each rat was 2 mins. For each area, tissues were pooled from two rats to constitute a sample. Samples weighed 80-100 mg for cortex and 30-50 mg for striatum. Tissue samples were immediately stored in liquid nitrogen until being assayed.

Corresponding tissue samples from control and cobalt implanted rats were taken together for biochemical analysis.

## 7. BIOCHEMICAL ASSAY OF HMPG AND HVA

### A. HMPG IN BRAIN

HMPG was estimated by the micromethod of Ashcroft et al (1975) which is used for tissues of 100 mg and less and is essentially based on that of Walter & Eccleston (1974). The micromethod, however, allows the simultaneous estimation of HMPG and either HVA or 5-HIAA in brain tissue samples and CSF.

In principle, the method for HMPG assay depends on hydrolysis of the conjugated HMPG with the enzyme 'Helicase' followed by extraction of the total HMPG with ethyl acetate and subsequent production of the electron capturing derivative, namely acetyl-trifluoracetyl-HMPG (acetyl-TFA-HMPG) for its quantitative estimation by gas liquid chromatography (GLC).

The maximum number of tissue samples that could be processed in one run was twenty. In every group of tissues to be assayed, two or four tissue samples were over 0.1 g in weight. The extracts of these heavier tissues were divided into two equal portions, one of which was used as a recovery for authentic HMPG.

### Reagents

The following reagents were used in the assay:-

0.4 M Perchloric acid (Analar), 1 M sodium acetate buffer, pH5, Ethyl acetate (CT grade), 1.65 M potassium bicarbonate (Analar), Dichloromethene (BDH redistilled), Anhydrous sodium sulphate (Analar), Acetic anhydride (Analar), Trifluoro-acetic anhydride (Sigma), 1 mg HMPG base/ml using 4-hydroxy-3-methoxyphenylglycol (HMPG)-bis-piperazine salt (Sigma), HCH hexachlorocyclohexane (HCH BDH), and Helicase (Industre Biologique Francaise). Acetic anhydride and some of the ethyl acetate (used for injection into the gas liquid chromatograph), were distilled before use, using a fractionation column.

Helicase is an enzyme preparation composed of a mixture of sulphatase ( $1.5 \times 10^7$  Roy Units/g) and glucuronidase ( $1 \times 10^6$  Fishman Units) and consisted of a lyophilized preparation of the gastric juice of *Helix pomatio*. The pH optimum of sulphatase was stated by the manufacturer to be 5. Using tritiated HMPG sulphate it was found that a mean of  $87\% \pm 9\%$  S.D. of HMPG sulphate was hydrolyzed by the enzyme and extracted into ethyl acetate in comparison to less than 2% extracted without the enzyme (Pullar, 1971).

Crude enzyme, as supplied, was found to give high background fluorescence during HMPG estimation which was markedly reduced by acetone purification (Pullar, 1971). Accordingly, the crude enzyme was purified by acetone as follows, before employing it for the GLC estimation of HMPG:-

One vial of Halicase was dissolved in about 30 ml distilled water and chilled in the fridge. Acetone (BDH) was also chilled. A magnetic follower was then placed in the 'Helicase' solution and about

300 ml of cold acetone was steadily poured into the agitated solution. The solution was then filtered immediately through a Buchner funnel and washed with further 100-200 ml of cold acetone. The precipitated enzyme was dried overnight in a dessicator over pellets of sodium hydroxide and then stored in the fridge.

A 50 mg/ml solution, freshly prepared, should be clear, and not cloudy, although brown. It was shown that the maximum activity of 9 mg/ml 'Helicase' solution was obtained after 8 hrs incubation (Walter, 1973). The activity of each enzyme preparation was ascertained and compared to that of a previously treated enzyme as described later, before being employed in the assay.

#### ASSAY PROCEDURE

The brain tissue samples were removed from liquid nitrogen on to a piece of weighted aluminium foil and the tissue weight quickly determined. The tissue was kept on a tray of solid CO<sub>2</sub> (-75°C) after weighing and before it was homogenized, to avoid its thawing.

##### 1. Homogenisation and precipitation of proteins and lipids

Tissues of 0.1 g and less in weight were homogenised in 1 ml of chilled 0.4 M perchloric acid in a chilled homogenizer (Jencon's). The homogenate was poured into a chilled 3.5 ml polythene centrifuge tube and the homogenizer washed with 0.2 ml of the acid which was added to the homogenate. All samples were placed on ice.

Tissues of more than 0.1 g, to be used for recovery, were

homogenized in the same way, but using 2 ml and 0.4 ml of perchloric acid, respectively.

The homogenate was centrifuged in an MSE high speed 18 centrifuge at 3,000 g for 10-15 mins at 4°C.

## 2. pH Adjustment

The supernatant was decanted into a 5 ml beaker, a 1 cm long magnetic follower added and the pH adjusted using a pH meter and a magnetic stirrer, to about 5-5.5 by the careful addition of 5 N, 1 N, and 0.02 N KOH. 1 N HCl was kept at hand for addition if too much KOH was added.

The solution was poured into a chilled conical (15 ml) stoppered test tube, the beaker washed out with a few drops of distilled water added to the extract in the tube.

The sample was then placed in the deep freezer for 15 - 50 mins to ensure maximum precipitation of potassium perchlorate. It was then allowed to thaw and centrifuged in an MSE Mistral at 3,000 g for 5 - 10 mins at 4°C. The supernatant was then decanted into a 15 ml conical test tube and the volume made up to 4 ml with distilled water.

## 3. Separation of HMPG and HVA portions

The solution was divided into two portions, each of 2 ml for the estimation of HMPG and HVA respectively. The HVA portion at about pH 5 was stored in the deep freezer overnight for subsequent assay.

#### 4. HMPG tissue recoveries

The supernatant volume from the heavier tissues of the occipital cortex, adjusted to 4 ml with distilled water as above, was divided into two equal portions. Standard amounts of 20 mg and 50 mg HMPG were added to one portion of each of such four or two extracts, respectively.

#### 4. Aqueous standard solutions

Standard HMPG solution of 20, 20 50 and 50 mg HMPG were freshly prepared, the volume of each being adjusted to 2 ml with distilled water. For the duplicate blanks, distilled water was used to a volume of 2 ml.

#### 6. Hydrolysis of conjugated HMPG

To each sample, 100  $\mu$ l of 1 M sodium acetate buffer pH 5, 50  $\mu$ l Helicase 50 mg/ml freshly prepared, and a drop of chloroform (to prevent bacterial growth), was added. The sample was incubated overnight in a water bath at 37°C for about 17 hrs.

#### 7. Extraction of glycol metabolites

Samples were taken out of the water bath. Each sample, of pH about 5, was shaken twice for 5 mins each time, with 4 ml and 3 ml ethyl acetate (CT grade). The layers were separated by centrifugation, and 3 ml and 3.5 ml respectively, of the ethyl acetate layer, which contains the glycols, were removed into a 10 ml glass test tube. The pooled ethyl acetate extracts were evaporated to dryness in the test tube by blowing nitrogen in a

heated block at 56°C.

8. Acetylation of glycols

The residue was taken up in 0.4 ml water and acetylated by the addition of 50 µl acetic anhydride (redistilled) and 0.6 ml of 1.65 M potassium biocarbonate solution, and the reaction allowed to proceed for 35 mins. The acetylated HMPG was extracted by shaking for 1 min with 1.5 ml dichloromethane followed by centrifuging.

9. Achieving anhydrous conditions

1.3 ml of the lower organic layer (containing the acetylated derivatives of the alcohol metabolites) was shaken in an Ependorff tube, with a little anhydrous sodium sulphate. The dichloromethane extract was carefully decanted into a 3 ml hard glass test tube and evaporated by blowing nitrogen at 56°C.

10. Trifluoroacetylation

The residue was taken up in 0.5 ml trifluoroacetic (TFA) anhydride mixture (prepared by mixing 1 part TFA plus 5 parts ethyl acetate (CT), prepared fresh).

The test tube is then stoppered and heated at 56°C for 15 min to convert the acetylated HMPG into its fluorinated derivative. After this 15 min, the solution was evaporated carefully to dryness under nitrogen at 56°C.

11. Addition of internal standard

Using Pasteur glass pipette, the residue was taken up in 0.2 ml ethyl acetate (CT grade, redistilled) containing 15 ng/ml hexachlorocyclohexane (HCH), as internal standard. The solution was transferred by Pasteur pipette into a GLC micro-vial. This can be stored for several days in the refrigerator before being put on the machine, as long as the vial cap is firmly closed.

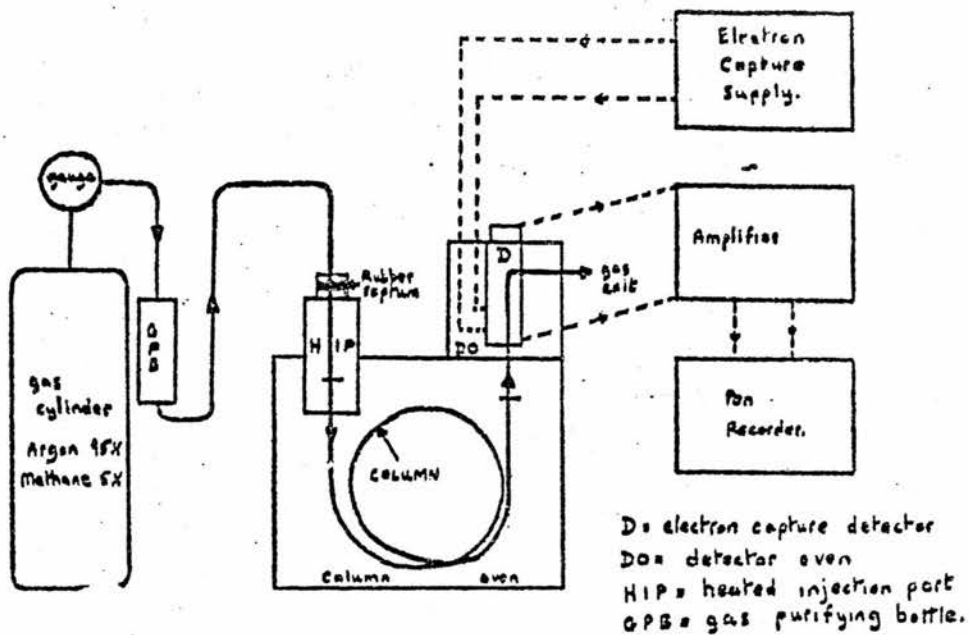
12. The quantitative estimation of HMPG by gas liquid chromatography (GLC)

In the present work, the procedure was performed using a Perkin-Elmer 900 gas liquid chromatograph fitted with a  $^{63}\text{Ni}$  electron-capture detector (Fig. 6). The carrier gas was Argon/methane (90%/10%) flowing in one eighth inch copper piping at a pressure of 70 lb/sq inch. The pressure in the cylinder head should be at least 10 lb/sq inch greater than the pressure setting in the GLC, and it was usually set at 100 lb/sq inch.

The injection head was screwed on the machine with a gas-tight joint, to a 5 ft long Perkin-Elmer column, 4 mm internal diameter. The stationary phase was 2.5% methyl-silicone gum rubber (E 301) on a chromosorb support (GAW-DMCS) 80-100 mesh. The powder was kept in position with a spun glass yarn plug. The flow rate in the GLC was measured as 60 ml/min with a soap bubble flow meter at exit.

Samples were introduced into the system by on-column injection of the ethyl acetate solution using 10  $\mu\text{l}$  glass micro-syringe with a 5 cm long needle. An automatic injection device (Hewlett-Packard) delivering 2  $\mu\text{l}$  or 4  $\mu\text{l}$  was used with minimum





**FIG 6**

A schematic diagram showing the experimental system for gas chromatography. The column oven and detector oven power supply and temperature control units have been omitted for the sake of clarity.

wash-out. The time between the injection of consecutive samples was 60 min to allow for the maximum clearing of the gas from the machine, thus reducing the chance of contamination with vapours from the previous sample. The needle was pushed quickly through the rubber septum at the injection port head, the sample injected and the needle quickly withdrawn. The speed of the chart paper was set at 1 cm/min. The Perkin-Elmer electron-capture detector consisted of a cylindrical source of  $^{63}\text{Ni}$  at the periphery of a small chamber, through which the carrier gas and sample vapour passed surrounding a centrally-placed probe. The steady state current across the chambers from the source to the probe was maintained by electron capture supply unit using a direct current supply.

The final settings with the approximate temperature equivalents were as follows:-

	<u>Setting</u>	<u>Temperature (°C)</u>
ECD	2	295
Manifold	4	245
Column	165	170
Injection	5	250

The column temperature was set to give a retention time of 2.2 min and 3.8 min for HMPG and HCH (internal standard) respectively.

#### Calculations

For each sample, the height of each acetyl-TFA-HMPG and HCH peak (in min) was measured and the ratios of the former to the latter, is determined (Sharman, 1969).

Hence:-

$$\text{Amount of HMPG in each sample (ng)} = \frac{\text{sample ratio} \times \text{recovery} \times 10}{\text{mean ratio equivalent to 10 ng HMPG in aqueous standard solution}}$$

$$\text{HMPG concentration (ng/g)} = \frac{\text{Amount of HMPG in samples (ng)}}{\text{tissue weight (g)}}$$

#### HVA IN BRAIN

The concentration of HVA in brain tissue was estimated by the micromethod (Ashcroft et al, 1975). The method requires a minimum weight of 15 mg of striatal tissue for estimation of the metabolites. In principle, the method is based on extraction of HVA into butyl acetate and followed by back extraction into 0.15 M sodium borate buffer and subsequent fluorophore production.

#### Reagents

1. Redistilled butyl acetate (BDH reagent grade) saturated with water.
2. Ascorbic acid 3 mg/ml, freshly prepared.
3. 2 N HCL with 87 ml of concentrated HCL (about 13 N) were made up to 500 ml with distilled water.
4. 0.15 M borate buffer, pH 8.5 - Add 1 M NaOH (4 g in 100 ml distilled water) to 0.15 M boric acid (9.28 g/l) until the pH reached 8.6. 250 ml were prepared at a time and stored in the refrigerator.
5. Alkaline potassium ferricyanide ( $\text{K}_3\text{Fe}(\text{CN})_6$ ), freshly prepared. A fresh solution containing 11 mg  $\text{K}_3\text{Fe}$

(CN)<sub>6</sub>/ml was prepared. Before use, 12.2 ml concentrated ammonia (BDH containing 35% NH<sub>3</sub>) was mixed with 12.8 ml distilled water and then 100 µl K<sub>3</sub>Fe (CN)<sub>6</sub> (11 mg/ml) was added to the mixture.

6. Cysteine solution, 1.75 mg/ml, was prepared freshly before use.

7. Homovanillic acid (HVA) (Calbiochem, A grade), stock standard, 1 mg/ml, in distilled water, was prepared and stored in the refrigerator. This was renewed every 4 weeks.

#### ASSAY PROCEDURE

##### 1. Extraction of HVA

To the 2 ml extract of tissue samples in a 15 ml conical test tube, 25 µl ascorbic acid (2 mg/ml) and 50 µl of 2 N HCl were added and the solution saturated with sodium chloride. The acid metabolites were extracted by shaking twice, for 5 min each time, with 5 ml redistilled butyl acetate, saturated with water. The samples were then centrifuged and 4 ml and 5 ml of the upper butyl acetate layer removed into another 15 ml conical test tube. The acids were then re-extracted into 0.7 ml of 0.15 M borate buffer, pH 8.5, by shaking for 5 min. The samples were centrifuged and the upper butyl acetate layer aspirated off using a water pump.

0.5 ml of the lower aqueous layer was then taken with Eppendorf pipette for the assay of HVA.

Aqueous standard solutions containing 20, 50, 100 and

200 ng HVA, respectively and in duplicates, were extracted and assayed with each batch of samples. Recovery samples were not required in HVA assay in different brain regions due to the high recovery of added HVA taken through the method.

## 2. HVA fluorophore production

To each 0.5 ml sample in an Eppendorff tube, 200  $\mu$ l alkaline  $K_3Fe(CN)_6$  solution was added. Exactly 4 min later the reaction was inhibited by the addition of 50  $\mu$ l cysteine solution (1.75 mg/ml).

## 3. Spectrophotofluorimetric assay of HVA

The fluorescence was read on a Perkin-Elmer spectrophotofluorimeter MPF-3A within 1 - 2 hours, using an excitation slit of 2 and an emission slit of 16. No filter was required. The emission wavelength was set at 418 nm and the excitation scan run between 250 nm and 380 nm. The fluorescence due to HVA fluorophore was measured by the vertical distance from the top of the peak at an excitation wavelength of 320-325 nm to a line drawn between two depressions at about 280 nm and 350 nm respectively.

## Calculations

For each tissue sample, the peak height of HVA fluorophore was measured (in mm) at 320 nm. The peak height due aqueous standard amount of HVA taken through the same method was also measured. From the latter, the average height equivalent (in mm) for 10 ng HVA was

calculated. Hence:-

$$\text{Amount of HVA in each tissue sample (ng)} = \frac{\text{Sample peak height} \times 10}{\text{Peak height equivalent for 10 ng in aqueous solution HVA.}}$$

Knowing the tissue weight, the concentration of HVA in the tissue sample could then be calculated:-

$$\text{HVA conc. (ng/g wet tissue weight)} = \frac{\text{Amount of HVA (ng)}}{\text{Tissue weight (g)}}$$

## 8. EXPERIMENTAL DESIGN

### A. LINEARITY OF THE MICROMETHOD FOR HMPG AND HVA

1. Determination in pure solutions, of the linearity of the electron capturing derivative formation of HMPG for estimation by GLC.

Ten solutions, each of 2 ml and containing 10 ng, 20 ng, 50 ng and 100 ng HMPG in duplicates, respectively, were freshly prepared. To each sample, 100  $\mu$ l sodium acetate buffer, pH 5.0 50  $\mu$ l of Helicase solution (50 mg/ml, freshly prepared) and one drop of chloroform were added. All samples were then incubated overnight at 37°C for the subsequent extraction, acetylation and trifluoroacetylation of HMPG and consequent GLC estimation as described above.

2. Tissue recovery of added HMPG processed through the micromethod

The brain of a male PGV rat was quickly dissected out and weighed after separation of cerebellum. Homogenization with 0.4 M perchloric acid (1 ml/100 mg) was done, as described. After pH

adjustment with potassium hydroxide and centrifugation, the volume of the supernatant was about 22.5 ml which was divided into ten portions each of 2.2 ml. Standard amounts of 10, 20, 50 and 100 mg HMPG, freshly prepared, were added to these portions, in duplicates, respectively. The remaining portions served as tissue standard duplicates. To each sample 100  $\mu$ l of 1 M acetate buffer pH 5, 50  $\mu$ l of freshly prepared helicase, 50 mg/ml and 1 drop chloroform were added. Samples were then incubated overnight (17 hrs) in a water bath at 37°C for subsequent extraction and estimation of HMPG by GLC as described before.

Calculation of the per cent recovery of HMPG was done as follows:-

The ratio of acetyl-TFA HMPG peak to that of HCH was determined for each sample. The average ratio equivalent to 10 ng HMPG added to brain tissue samples was determined. Similarly, the average ratio equivalent to 10 mg HMPG in aqueous standard solution calculated. Hence:-

$$\begin{array}{lcl} \% \text{ recovery of} & = & \frac{\text{Ratio equivalent to 10 ng HMPG added} \times 10}{\text{Ratio equivalent to 10 ng HMPG in aqueous}} \\ \text{added HMPG} & & \text{standard solution.} \end{array}$$

### 3. Determination, in pure solution, of the linearity of HVA fluorophore formation

Ten solutions, each of 2 ml and containing standard amounts of 20, 50 and 100 ng HVA in duplicates respectively, were freshly prepared.

All samples were then taken for HVA extraction twice with

butyl acetate, back extraction with borate and eventual fluorophore formation as described before.

#### 4. Tissue recovery of added HVA processed through the micromethod

The striatum from 5 male PVG rats, about 12 weeks old, was quickly dissected out after decapitation and pooled before being weighed. Homogenization was done with 0.4 M perchloric acid using 1 ml for each 100 mg tissue weight as described before.

After pH adjustment with potassium hydroxide, the supernatant volume was 17.2 ml, and it was divided into 8 portions each of 2.1 ml. To these portions standard amounts of 20, 50 and 100 ng HVA standard were added in duplicates, respectively. The remaining portions served as tissue standard duplicates. All samples were then subjected for HVA extraction and estimation as described above. Determination of % recovery of HVA added to brain tissue was done as follows:-

The HVA fluorophore peak height (in mm measured at 320 nm) was measured for each sample. The mean peak height (in mm) equivalent to 10 ng HVA added to brain tissue was determined. Similarly, the mean peak height (in mm) equivalent to 10 ng HVA in aqueous standard solution was calculated. Hence:-



$$\% \text{ recovery of added HVA to brain tissues} = \frac{\text{Peak height (in mm) equivalent to 10 ng HVA added to brain tissue}}{\text{Peak height (in mm) equivalent to 10 ng in aqueous standard solution.}} \times 100$$

#### SPECIES VARIATION OF HMPG AND HVA.

Ten male Wistar rats, 12 weeks old and weighing 180 - 250 g, and 10 male PVG rats, of similar age and weight, were used. From each rat the striatum was dissected on each side and portions of frontal cortex were taken from each side after decapitation as described before.

From each side, frontal cortex or striatum was pooled from two rats to constitute a tissue sample for that region. Thus, 5 samples of frontal cortex or striatum were obtained for the right and left sides from ten rats in each group. Portions of occipital cortex were also pooled from 6 rats for estimation of tissue recovery of HMPG. Frontal cortex samples, recovery samples, as well as freshly prepared aqueous standards of HMPG were taken through the micromethod for the estimation of HMPG levels by GLC as described above.

Tissue samples from striatum, together with freshly prepared aqueous solutions containing standard amounts of HVA were taken through the micromethod for assay of HVA levels as described above.

#### B. HMPG AND HVA LEVELS IN COBALT-IMPLANTED RATS

1. HMPG and HVA levels in unoperated and sham operated controls 8 days after operation

Ten PVG rats were used in the experiment of which 5 were operated controls.

Under halothane anaesthesia, these rats were operated upon exactly like cobalt-implanted litter mates, but after trephining the skull with No. 6 burr over the right frontal cortex 2 mm in front of coronal suture and 2 mm lateral to the sagittal suture, these rats received no cobalt implant. The hole in the skull was closed with bore wax and after spraying the operative held with polybacterin, the skin was sutured with silk.

No special post-operative care was necessary. None of the animals displayed clinical jerks in the post-operative period. At time of sacrifice, 8 days after operation, the rats were killed by decapitation and their brain quickly removed on solid CO<sub>2</sub> and crushed ice mixture. Portions of the frontal cortex and striatum from each side were quickly dissected and taken to constitute a sample. No pooling of tissues was done. Tissues were quickly stored in liquid nitrogen. Thus, five samples from each cortex were available for HMPG estimation and five samples from each striatum for HVA assay from the 5 sham operated rats. A similar number of samples for cortex and striatum were taken from the 5 unoperated control rats.

Samples were assayed within 3-4 days of storage in liquid nitrogen. Samples of both groups of rats were taken together for biochemical assay.

2. HMPG and HVA levels at  
8 days after cobalt implantation

Thirty-two PVG rats were used of which 16 animals served as unoperated controls.

Under halothane anaesthesia, 16 of these rats received cobalt implants as described previously. No special post-operative care was necessary.

Eight days after implantation, when contralateral forelimb jerks and whisker twitches were well established, the rats were killed by decapitation, and their brains quickly dissected out and put on a mixture of solid CO<sub>2</sub> and crushed ice. After the cerebellum was removed, tissue portions were taken from the right frontal cortex (RFC) (after enucleating any excess cobalt and, sometimes, calcium deposits), left frontal cortex (which is the site of mirror focus), the right striatum (RS), left striatum (LS) and occipital cortex (OC). Tissues were pooled from 2 rats to constitute a sample for the particular area.

Control rats were treated in the same way after decapitation. Thus, 8 tissue samples were available for each region studied in 16 cobalt implanted rats and a similar number from 16 unoperated controls. Tissue samples from both groups of animals were taken together for homogenization and biochemical estimation of HMPG and HVA.

3. HMPG and HVA levels at 3, 7 and 11 days after cobalt  
implantation in PVG rats

The absence of any significant changes in the major cate-

choline metabolite levels 8 days after cobalt implantation, when epileptic manifestations were marked, contrasted with the changes in these metabolites previously reported. Thus, it was desirable to follow the changes in these metabolites as indices of their parent catecholamine formation and turnover at different stages of development of focal epilepsy in cobalt implanted rats.

This was done at the following time periods:-

A. 3 days after cobalt implantation.

At this time, both primary and secondary foci were early in development.

B. 7 days after cobalt implantation.

The epileptic activity from both foci was maximal.

C. 11 days after cobalt implantation.

The epileptic activity from both foci was still prominent.

For this purpose, sixty male PVG rats were operated upon, of which twenty-four rats served as sham operated controls.

Of the thirty-six cobalt-implanted animals, eighteen rats received cobalt implant into the right frontal cortex and had four recording screw electrodes fixed to the skull, while the remaining rats had only cobalt implants into the right frontal cortex.

Of the 24 sham control rats, 12 animals had the dura mater over the right frontal cortex incised and 4 recording screw electrodes fixed to the skull, while the remaining rats had only the dura mater over the right frontal cortex incised without the fixation of any screw electrodes.

The operative procedure for cobalt implantation and the fixation of screw electrodes is already described in the Methods.

Electrocorticographic (ECoG) recording was done from rats with fixed electrodes at 3, 5, 7, 9 and 11 days after cobalt implantation. Each ECoG recording was for 10 min and simultaneously recorded on a magnetic tape (Tandberg Series 100) for computer spike analysis (Hill & Townsend, 1973). The ECoG instrumentation, recording technique and spike analysis is described in Section II (Methods).

Rats were sacrificed at 3, 7 and 11 days after cobalt implantation. During each of these days, 12 cobalt implanted rats were sacrificed, half of which had recording screws fixed to the skull, while 8 sham controls were killed, half of which were with electrodes.

After decapitation, the brain of each rat was quickly dissected out onto solid CO<sub>2</sub> and crushed ice mixture and cerebellum removed. Tissue portions were taken, using sterile forceps, from right frontal cortex (RFC) which is the site of the primary focus, left frontal cortex (LFC) which is the site of the

secondary focus, the right striatum (RS), left striatum (LS), and occipital cortex (OcC). The technique of dissection is described in the Methods.

Tissues were pooled from each of two rats in a group to constitute a sample for each of the above brain regions, one rat with electrode and the other without electrode.

Thus, 6 samples were available for each of the above brain regions from 12 cobalt implanted rats sacrificed on each of the days mentioned above in contrast to 4 samples for each brain region from 8 sham operated controls sacrificed at the same time.

The frozen samples on solid CO<sub>2</sub> were taken quickly for storage in liquid nitrogen. Subsequent biochemical analysis was completed within 3-4 weeks of storage.

C. STUDY OF THE POST-MORTEM STABILITY OF HMPG AND HVA IN BRAIN TISSUE.

The usual method of handling rat brain tissues was to dissect the brain out quickly onto solid CO<sub>2</sub> (-75°C) after decapitation and to take the brain tissue as soon as possible to liquid nitrogen storage (-195°C) until the beginning of the assay (Clayton, 1976).

At the time of the assay, the brain tissue was taken out of liquid nitrogen onto solid CO<sub>2</sub>, weighed and then homogenized for the estimation of HMPG and HVA. So, two time periods were involved, namely:-

- A. The time between death and storage in liquid nitrogen (-195°C), i.e. before freezing.

- B. The time between the removal of brain tissue from liquid nitrogen ( $-195^{\circ}\text{C}$ ) and the starting of the assay, i.e. after freezing.

Since HMPG and HVA are formed by the enzymatic degradation of noradrenaline and dopamine respectively, it was conceivable then that changes in environmental temperature as well as the duration of time required for each step of tissue handling might alter the rate of formation and consequently the concentration of these metabolites in the brain. So, it was desirable to investigate the effect of these factors, namely, time and temperature, on the brain concentration of both metabolites.

For both time periods mentioned above, changes in environmental conditions were induced by exposing the brain tissue to:-

1. Immediate homogenization and assay.
2. Solid  $\text{CO}_2$  ( $-75^{\circ}\text{C}$ ).
3. Ice ( $0^{\circ}\text{C}$ ).
4. Room temperature ( $20^{\circ}\text{C}$ ).

#### I. HMPG POST MORTEM STABILITY

##### A. BEFORE FREEZING IN LIQUID NITROGEN

Twenty PVG rats were used. As mentioned above, variation in environmental conditions were instituted by exposing the brain tissue to the following:-

1. Homogenized immediately for assay.
2. Immediately to liquid nitrogen storage ( $-195^{\circ}\text{C}$ ).
3. Ice ( $0^{\circ}\text{C}$ ) for 30 min. Tissue from frontal cortex was put on ice for 30 min before being taken quickly to nitrogen storage.
4. Room temperature ( $20^{\circ}\text{C}$ ) for 30 min.

The decapitated head of the rat was put on the bench for 30 min before dissecting the brain tissue quickly for storage in liquid nitrogen.

Each tissue sample was obtained by pooling portions of the frontal cortex on each side of one rat. Each tissue sample weighed at least 200 mg before assay for duplicate estimates of HMPG, as described above.

For each condition, five tissue samples were assayed from the rats used in the experiment.

#### B. AFTER FREEZING IN LIQUID NITROGEN

Twelve PVG rats were used. Pooled frontal cortex, weighing at least 600 mg taken from the brain of 2 decapitated PVG rats was distributed into three polythene bags and quickly placed in liquid nitrogen. Before the start of the assay, these tissue samples were taken out of liquid nitrogen storage and each sample was exposed to a different environmental condition as follows, before homogenization:-



1. Solid CO<sub>2</sub> (-75°C) for 5 min.
2. Ice (0°C) for 5 min.
3. Room temperature (20°C) for 5 min.

Here the tissue sample was put on the bench.

Each sample weighed at least 200 mg before assay. Thus, for each condition, six tissue samples were processed from the rats used in the experiment.

After homogenization and centrifugation and pH adjustment, duplicate estimates of HMPG were made on the supernatant of each tissue sample as described before.

In all HMPG assays, duplicate aqueous standards of 10, 20, 50 and 100 ng HMPG, respectively in buffer and with Helicase, were processed with the above tissue samples.

For tissue recoveries, frontal cortex was pooled from 2 rats and homogenized with perchloric acid for duplicate recoveries of 10, 20, 50 and 100 ng HMPG respectively.

## II. HVA POST-MORTEM STABILITY

### A. BEFORE FREEZING IN LIQUID NITROGEN

Twenty male PVG rats were used. After dissection, the striatum was exposed to the following conditions, as with HMPG:-

1. Homogenized immediately for assay.
2. Immediately to liquid nitrogen storage (-195°C).

3. Ice (0°C) for 30 min before being taken to liquid nitrogen.

4. Room temperature (20°C) for 30 min.

The decapitated head was put on the bench for 30 min before dissecting the striatum quickly out for storage in liquid nitrogen.

For each condition, 5 tissue samples were used. Each tissue sample was obtained by pooling the right and left striatum of one male PVG rat after decapitation and dissecting the brain out. Each tissue sample weighed between 40-50 mg, before assay.

After homogenization and centrifugation and pH adjustment, the supernatant of each sample was assayed for duplicate estimate of HVA as described above.

#### B. AFTER FREEZING IN LIQUID NITROGEN

Fifteen PVG rats were used. The right and left striatum were pooled from 3 rats, weighing between 120-140 mg and quickly distributed into 3 polythene bags for each condition. Samples were quickly frozen in liquid nitrogen.

At the time of assay, tissue samples were taken out of liquid nitrogen, weighed, and exposed to the following conditions before homogenization:-

1. Solid CO<sub>2</sub> (-75°C) for 5 min.
2. Ice (0°C) for 5 min.
3. Room temperature (20°C) for 5 min.

Thus, for each condition, 5 tissue samples were obtained from rats used in the experiment. Each sample was assayed for duplicate estimates of HVA as described above.

For all HVA assays, duplicate aqueous standards of 30, 40, 50 and 100 ng HVA respectively, were processed with the above samples.

### Precision

An estimate of precision was obtained from a consideration of the differences between duplicate estimations. This is expressed as standard derivation,  $S.D. = \sqrt{\frac{\sum d^2}{2N}}$  Where d is the difference between two results in a duplicate determination and N is the number of duplicate determinations performed. Furthermore, % variability between duplicates was determined as follows:-

$$\begin{array}{lcl} \text{\% variability} & & \text{Differences between the two results} \\ \text{between any duplicate} & & \text{in the duplicate estimations} \\ \text{estimations} & = & \frac{\text{the average concentration of the}}{\text{duplicate estimate}} \times 100 \end{array}$$

$$\begin{array}{lcl} \text{and the mean \% variability} & & \text{Total \% variability} \\ \text{between duplicates in a} & & \text{N (number of duplicates)} \\ \text{group} & = & \text{in a group.} \end{array} \times 100$$

## RESULTS

In the GLC estimation of HMPG by the micromethod, and under the GLC settings described in the method, acetyl-TFA-HMPG and HCH retention occurred at 2.2 and 3.8 min, respectively, after the injection (See Fig. 7).

In pure solution, the formation of the derivative acetyl-TFA-HMPG was linear with different amounts of HMPG. A linear calibration graph was obtained when aqueous standard amounts of 10, 20, 50 and 100 ng HMPG were assayed (Fig. 8).

The formation of the electron capturing derivative, acetyl-TFA-HMPG was also linear when different amounts of HMPG were added to brain tissue homogenates.

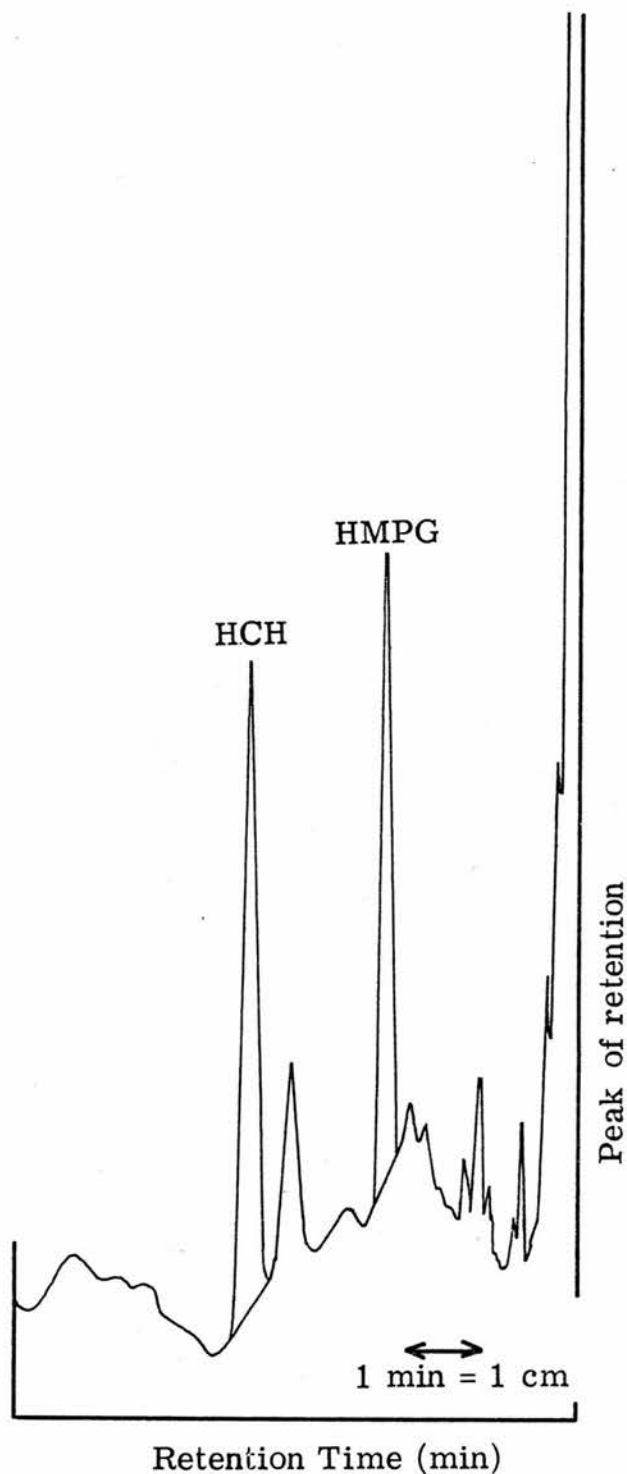
The results from assay of 10, 20, 50 and 100 ng HMPG added to supernatant from brain tissue homogenates are shown in Table 1.

The % recovery of added HMPG to brain extracts was 77.4%.

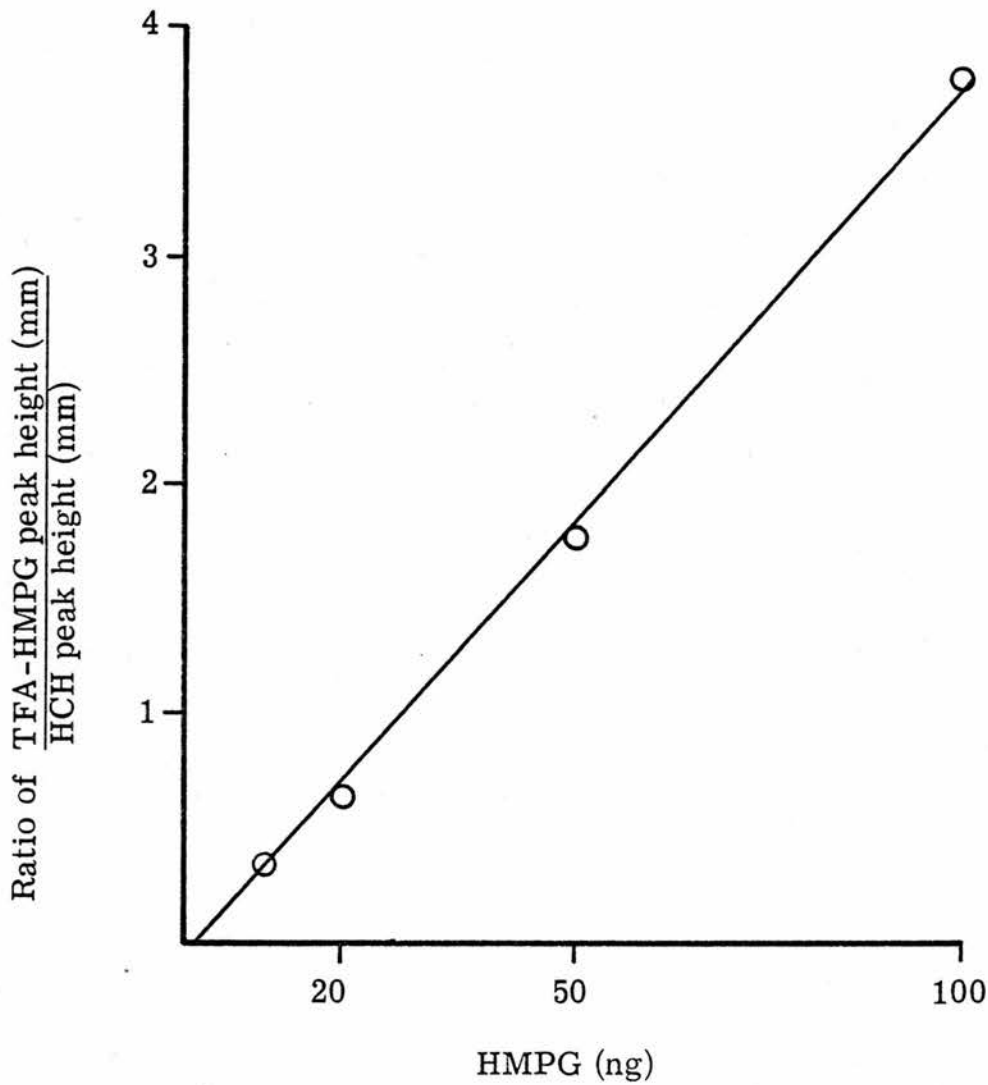
The sensitivity of the method was equivalent to 2 ng HMPG per tissue sample, (which is equivalent to 200 ng/g of wet tissue weight).

In the estimation of HVA by the micromethod and subsequent spectrofluorimetry, the peak of HVA fluorophore appeared at 320 nm and the height of the peak measured as previously described (Fig. 9).

In pure solutions, HVA fluorophore formation was linear with different amounts of HVA standards over the range of 20-100 ng (Fig. 10).



Retention of acetyl-TFA-HMPG, and that of the internal standard, HCH. Retention at 2.2 and 3.8 min. respectively, as detected by the Perkin-Elmer electron capture detector of the GLC (column temperature  $170^{\circ}\text{C}$ ). Gas flow was 60 ml/min. Paper speed = 1 cm/min. HMPG and HCH retentions are measured by the vertical distance from the top of either peak to a line drawn between two depressions at the beginning and end of each retention, and constituting the base of each peak respectively.

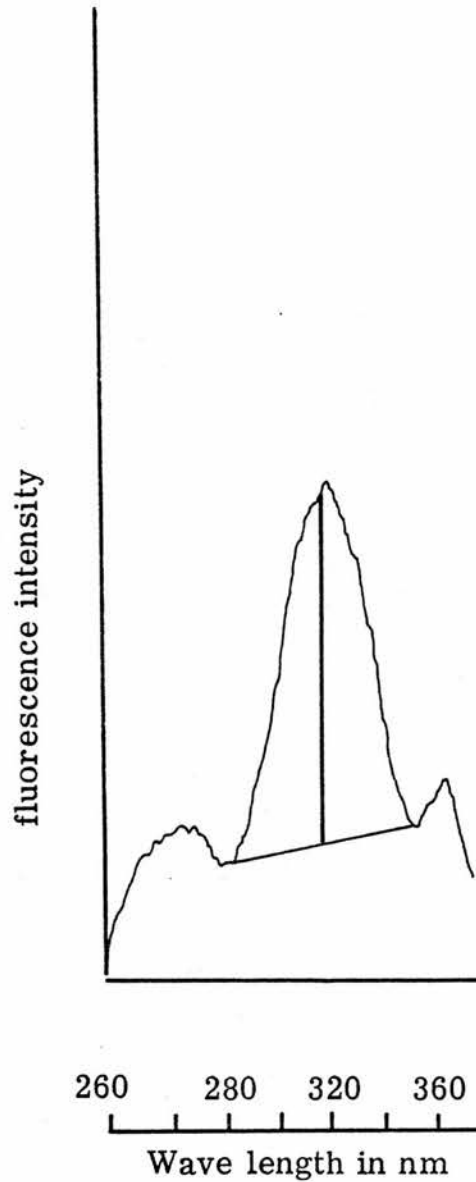


Showing the linear relation between the different concentrations of HMPG and the formation of acetyl-TFA derivative of HMPG as measured by the ratio of the peak height of the derivative to that of the internal standard, HCH. The line is a regression line. Each point represents the average of two determinations

TABLE 1. THE RECOVERY OF HMPG ADDED TO POOLED RAT BRAIN AND TAKEN THROUGH THE MICROMETHOD FOR EXTRACTION, PURIFICATION AND ACETYL-TFA DERIVATIVE FORMATION

Sample	Acetyl-TFA HMPG peak height (mm)	HCH peak height (mm)	Ratio of HMPG peak HCH peak	Ratio equivalent to the amount of HMPG added to tissue homogenate	Ratio equivalent to 10 ng HMPG added to tissue homogenate
Tissue Standard	123	53	2.32		
"	118	50	2.36		
Tissue + 10 ng HMPG	125	48	2.60	0.26	0.26
" + "	141	52	2.61	0.27	0.27
Tissue + 20 ng HMPG	138	48	2.88	0.54	0.27
" + "	145	50	2.90	0.56	0.28
Tissue + 50 ng HMPG	176	48	3.67	1.33	0.26
" + "	165	46	3.58	1.24	0.25
Tissue + 100 ng HMPG	157	32	4.90	2.56	0.256
" + "	152	32	4.75	2.41	0.241

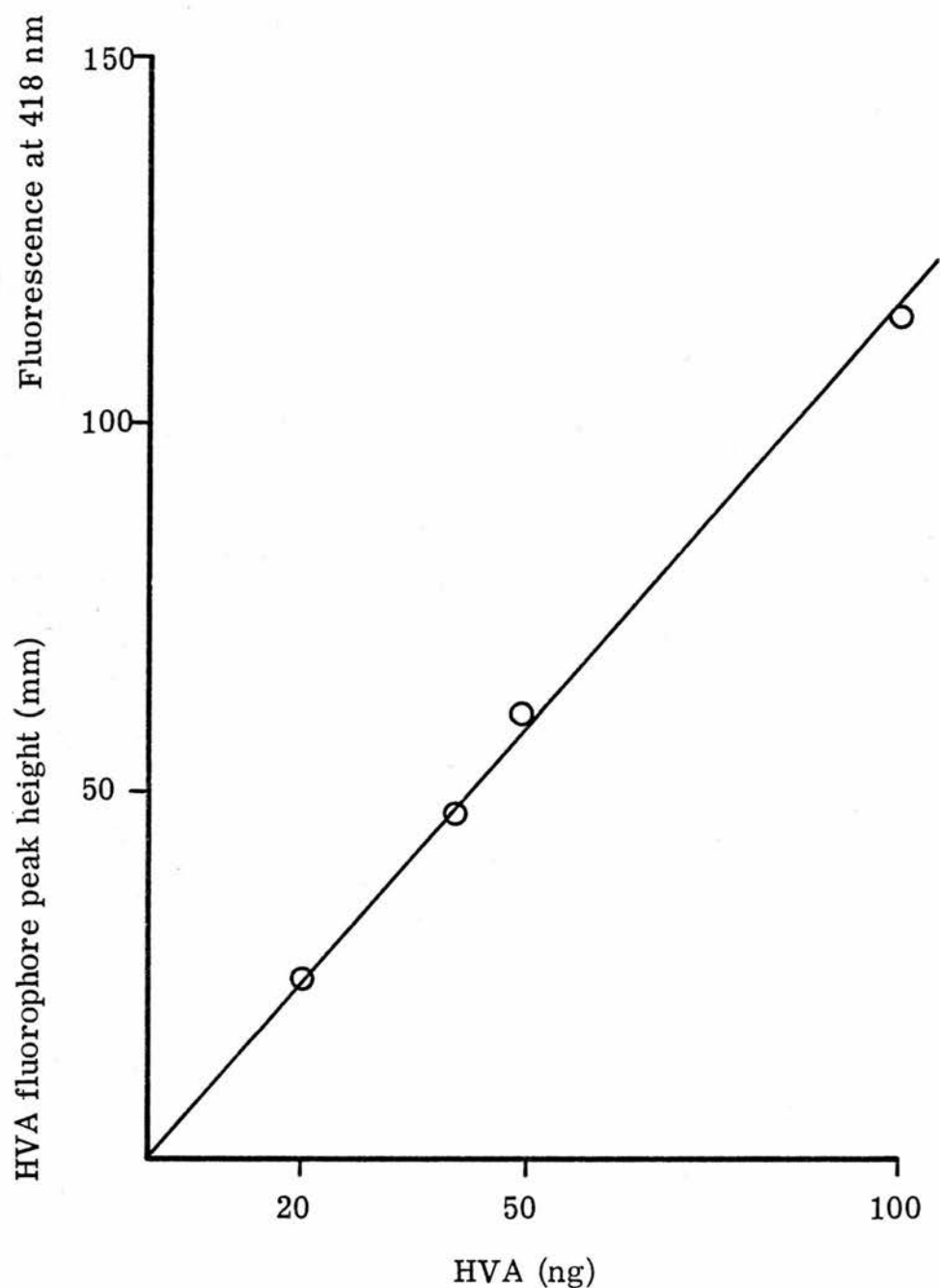
The table above shows the linear ratio between the starting concentration of HMPG and the ratio of Acetyl-TFA HMPG peak height to HCH peak height.  
Each 10 ng HMPG added to tissue brain homogenates is equivalent to a ratio of 0.261.  
Each 10 ng HMPG in pure solution was equivalent to a ratio of 0.337 (see Figure 8).  
% Recovery of added HMPG = 77.4%.



Activation spectrum (between 260 and 360 nm) of the fluorescence due to HVA fluorophore in extracts of striatum, as read in Perkin-Elmer MPF-3A, spectrophotofluorimeter. Emission wavelength is set at 418 nm. The fluorescence of the HVA fluorophore is measured by the vertical distance from the top of the peak, at an excitation between two depressions at about 280 and 350 nm respectively.



Fig 10



Showing the linear relation between the different concentration of HVA and the formation of HVA fluorophore, as measured by the spectrofluorimetric peak height of the fluorophore at 320 nm. The line is a regression line. Each point represents the average of two determinations.

TABLE 2

THE RECOVERY OF ADDED HVA TO POOLED RAT STRIATUM AND TAKEN THROUGH THE MICROMETHOD FOR EXTRACTION AND HVA FLUOROPHORE FORMATION

Sample	HVA fluorophore peak height (in mm) measured at 320 nm in spectrofluorimetric scan	HVA fluorophore peak height (in mm) equivalent to amount of HVA added to brain extracts
Tissue Standard	88	-
" "	92	-
Tissue + 20 ng HVA	112	22
" + "	114	24
Tissue + 40 ng HVA	134	44
" + "	138	48
Tissue + 50 ng HVA	146	56
" + "	150	60
Tissue + 100 ng HVA	200	113
" + "	207	117

The table above shows a linear relation of the extraction procedure coupled with fluorophore formation for different amounts of HVA added to brain homogenates.

Each 10 ng HVA added to tissue extract is equivalent to an average peak height of 11.48 mm. In aqueous solutions 10 ng HVA is equivalent to an average peak height of 11.9 mm (see Figure 10).

% recovery of HVA added to brain homogenates = 96%.

Similar linearity was obtained with brain tissue homogenate to which was added varying standard amounts of 20, 50 and 100 ng HVA, respectively (Table 2).

The % recovery of HVA added to brain extracts was 96%.

The sensitivity of the method was 20 ng HVA per tissue sample, which is equivalent to 400 ng/g and 300 ng/g wet tissue weight for cortex and striatum, respectively.

Since the recovery of HVA was so high, no correction of concentrations for recovery was made in subsequent assays and this is one of the advantages of the method.

Furthermore, employing the micromethod, the concentration of either HMPG and HVA in the frontal cortex or striatum respectively, of either Wistar or PVG rats, were not statistically significant ( $P = 0.5 - 0.6$ , and  $P = 0.4 - 0.5$ , respectively). (Tables 3A and 3B). The reported normal level of HMPG in the rat frontal cortex was 68 ng/g tissue weight ( $\pm 23$  S.D.) (Braestrup & Nielsen, 1972, Yates - unpublished data), while that of HVA in striatum was 700 ng/g tissue weight  $\pm 150$  S.D. (Westerink & Korf, 1975).

#### HMPG AND HVA LEVELS IN COBALT IMPLANTED RATS

The controls used for biochemical assays in cobalt implanted rats were either unoperated or sham operated control rats.

On the 8th post-operative day in sham-operated control rats, HMPG concentrations in the frontal cortex on both sides were not significantly different when compared to those of unoperated controls. (Table 4A). Furthermore, HVA concentrations in the striatum were not significantly different between both groups (Table 4B). Moreover, on the 8th day following cobalt implantation, HMPG concentrations (Table 5A) in the

TABLE 3A

HMPG CONCENTRATIONS IN FRONTAL CORTEX OF WISTAR AND PVG RATS

- values are expressed as ng/g wet tissue weight

Sample	Wistar Rats		PVG Rats	
	Right frontal cortex	Left frontal cortex	Right frontal cortex	Left frontal cortex
1	75	78	72	69
2	82	84	86	79
3	77	75	78	88
4	70	68	82	80
5	84	86	75	78
Mean	77	78	79	78
+ S.D.	6	8	5	6

The differences between both groups of rats were not statistically significant (Student's-t-test, two-tailed, with Bessel's correction)

TABLE 3B

HVA CONCENTRATIONS IN THE STRIATUM OF WISTAR AND PVG RATS  
 - values are expressed as ng/g wet tissue weight

Sample	Wistar Rats		PVG Rats	
	Right striatum	Left striatum	Right striatum	Left striatum
1	941	844	-	588
2	801	673	628	721
3	790	745	856	831
4	665	739	622	692
5	659	698	692	638
Mean	771	740	700	694
+ S.D.	104	59	94	82

The differences between both groups of rats were not statistically significant (Student's-t-test, two tailed, with Bessel's correction)

(-) = sample lost during the assay

TABLE 4A

HMPG CONCENTRATIONS IN FRONTAL CORTEX OF  
UNOPERATED AND SHAM OPERATED RATS ON THE  
8TH POST-OPERATIVE DAY

- values are expressed as ng/g wet tissue  
weight

Sample	Sham-operated Rats		Unoperated Rats	
	RFC	LFC	RFC	LFC
1	82	63	82	74
2	78	80	74	82
3	75	87	95	83
4	55	72	57	-
5	84	90	76	86
Mean	75	78	77	81
+ S.D.	12	11	14	5

RFC = Right frontal cortex

LFC = Left frontal cortex

The differences in respective concentrations  
between both groups of rats were not  
significant (Student's-t-test, two-tailed,  
with Bessel's correction)

(-) = sample lost during the assay

TABLE 4B

HVA CONCENTRATIONS IN THE STRIATUM OF  
UNOPERATED AND SHAM OPERATED RATS ON  
THE 8TH POST-OPERATIVE DAY

- values are expressed as ng/g wet tissue  
weight

Sample	Sham-operated Rats		Unoperated Rats	
	RS	LS	RS	LS
1	787	806	854	828
2	815	850	739	821
3	855	877	826	858
4	775	792	862	843
	-	-	-	-
Mean	811	831	828	838
+ S.D.	34	41	56	17

RS = Right striatum

LS = Left striatum

The differences in respective concentrations  
between both groups of rats were not  
statistically significant (Student's-t-test,  
two-tailed, with Bessel's correction)

(-) = sample lost during the assay

regions of the primary focus (right frontal cortex), secondary focus, left frontal cortex), and striatum on both sides were not significantly different when compared to controls, in spite of the tendency towards increases in the concentration in the treated rats. Similarly, the levels of HVA in the striatum on both sides showed a tendency towards reduction, but this was not significant when compared to controls (Table 5B). The absence of any changes in these metabolites contrasts with the marked motor epileptic manifestation experienced by the cobalt-implanted rats.

HMPG and HVA levels were also measured in cortical foci and striatum at different stages of development of epileptic in cobalt-implanted rats. The rats developed epileptic foci within 3 days of the application of cobalt to the right frontal cortex. The spike frequency of these foci was marked after 7-9 days, and was still prominent 11 days following cobalt implantation. Spike frequency was greater in the secondary focus than in the primary focus. Sham control rats showed no epileptic motor manifestations and no persistent ECoG changes. (Fig. 11).

HMPG and HVA levels were measured in the rat brain at 3, 7, and 11 days after the application of cobalt. The results of HMPG levels at these time periods are shown in Tables 6A, 7A and 8A, respectively.

At 3 days, the differences in HMPG levels in frontal cortex and striatum on both sides were not significantly different when compared to those of sham operated controls.



TABLE 5A

HMPG CONCENTRATIONS IN DIFFERENT BRAIN REGIONS 8 DAYS AFTER COBALT-IMPLANTATION INTO THE RIGHT FRONTAL CORTEX OF PVG RATS

- concentrations are expressed as ng/g wet tissue weight

S.D. = standard deviation

Sample	Cobalt-implanted Rats					Unoperated Controls Rats				
	RFC	LFC	RS	LS	OcC	RFC	LFC	RS	LS	OcC
1	103	95	150	131	57	55	53	-	83	70
2	89	135	120	103	80	-	88	88	96	54
3	88	75	71	106	67	89	92	69	86	50
4	-	114	-	-	95	87	89	68	-	92
5	90	123	108	130	68	78	87	92	95	88
6	98	103	110	-	82	-	-	-	-	-
Mean	94	108	112	118	75	77	82	79	90	71
+ S.D.	7	19	25	13	13	16	19	13	7	19

The differences in concentration between respective brain region of Cobalt-implanted and control rats were not statistically significant (Student's-t-test, two-tailed, with Bessel's correction)

RFC = Right frontal cortex (site of primary focus in Cobalt-implanted rats)

LFC = Left frontal cortex (site of secondary focus " " " " )

RS = Right striatum

LS = Left striatum

OcC = Occipital cortex

(-) = sample lost during the assay

TABLE 5B

HVA CONCENTRATIONS IN THE STRIATUM 8 DAYS AFTER COBALT-IMPLANTATION INTO THE RIGHT FRONTAL CORTEX IN PVG RATS

- concentrations are expressed as ng/g wet tissue weight

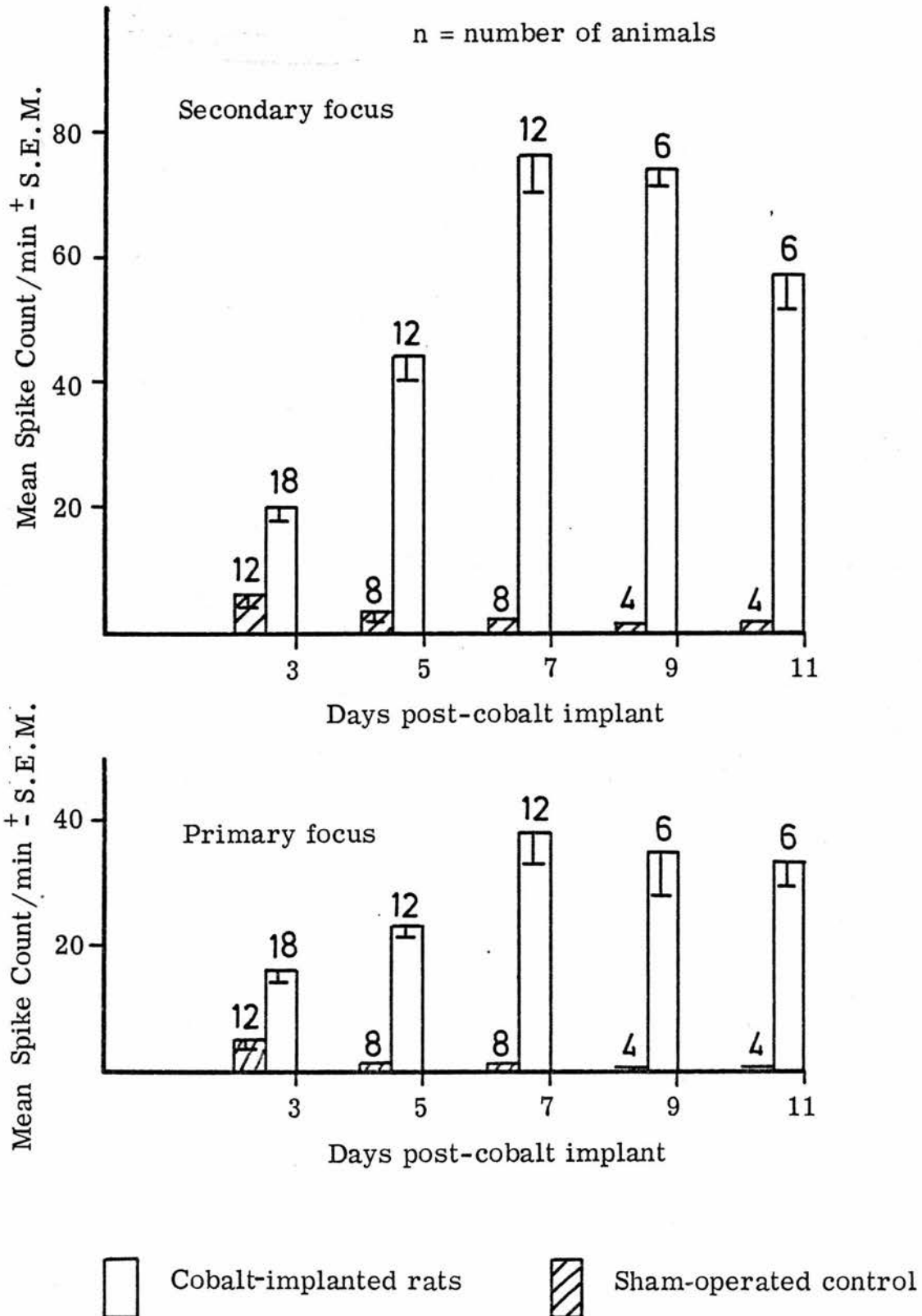
S.D. - standard deviation

Sample	8 Days Post-Cobalt Implantation				Unoperated Controls			
	RFC	LFC	RS	LS	RFC	LFC	RS	LS
1	N.D.	N.D.	807	-	N.D.	N.D.	889	941
2	"	"	921	839	"	"	869	-
3	"	"	605	940	"	"	821	864
4	"	"	674	727	"	"	752	743
5	"	"	816	753	"	"	854	841
6	"	"	796	68	"	"	806	-
Mean	N.D.	N.D.	770	788	N.D.	N.D.	831	847
+ S.D.	-	-	153	102	-	-	50	82

The differences in concentrations between respective brain regions in both groups of rats were not statistically significant (Student's-t-test, two-tailed, with Bessel's correction)

N.D. = Not detectable by the sensitivity of the method (i.e. below 20 ng/tissue sample)

Development of Epileptic Activity in Cortical Foci  
of Cobalt Implanted Rats



HMPG concentrations, however, tended to be higher in frontal cortex and striatum at 7 days, following cobalt implantation, but the differences were not significant when compared to those of control rats.

Moreover, no significant changes in HMPG levels between both groups of rats were found 11 days after cobalt implantation.

The results of HVA levels at similar time periods are shown in Table 6B, 7B and 8B.

There was no significant change in HVA levels in the striatum after 3 days of cobalt implantation in comparison to that in sham control rats.

The concentrations of HVA, however, tended to be lower in the striatum 8 days after cobalt application but the differences were not significant when compared to controls. Moreover, there was no significant change in HVA levels in striatum between both groups of rats 11 days after cobalt implantation. At these stages of development of epileptic foci, the variance of HVA levels was increased in cobalt implanted rats in comparison to controls. Furthermore, HVA could not be detected by the method in either the primary and secondary cortical foci during the different stages of their development. The normal concentration of HVA in the cortex is about 150 ng/g which is below that which could be detected by the sensitivity of the method. However, if there was an increase in dopamine turnover in these epileptic sites, it might be reflected by increased HVA levels within the range that could be estimated by the method.

TABLE 6A

HMPG CONCENTRATIONS IN DIFFERENT BRAIN REGIONS 3 DAYS FOLLOWING COBALT-IMPLANTATION INTO THE RIGHT FRONTAL CORTEX OF PVG RATS

- values are expressed as ng/g wet tissue weight

S.D. = standard deviation

Sample	3 Days Post-Cobalt Implantation					3 Days After Operation in Sham Controls				
	RFC	LFC	RS	LS	OcC	RFC	LFC	RS	LS	OcC
1	101	84	86	104	-	83	99	83	82	53
2	97	96	83	107	74	88	76	99	94	46
3	73	82	108	99	82	78	96	91	95	71
4	66	50	106	100	68	51	52	85	83	62
5	89	94	100	86	50					
Mean	85	81	97	99	69	75	81	89	88	58
+ S.D.	14	17	10	7	12	17	19	7	7	11

The differences in concentrations in respective brain regions of both groups of rats were not statistically significant (Student's-t-test, two-tailed, with Bessel's correction)

(-) = sample lost during the assay

TABLE 6B

HVA LEVELS IN THE STRIATUM 3 DAYS AFTER COBALT-IMPLANTATION INTO THE RIGHT FRONTAL CORTEX IN PVG RATS

- concentrations are expressed as ng/g wet tissue weight  
S.D. - standard deviation

Sample	3 Days Post-Cobalt Implantation				3 Days After Operation in Sham Controls			
	RFC	LFC	RS	LS	RFC	LFC	RS	LS
1	N.D.	N.D.	687	772	N.D.	N.D.	574	536
2	"	"	547	480	"	"	599	675
3	"	"	402	497	"	"	717	698
4	"	"	597	496	"	"	575	629
5	"	"	796	796				
Mean	N.D.	N.D.	606	608	N.D.	N.D.	615	634
+ S.D.	-	-	133	143	-	-	59	62

The differences in concentrations between respective brain regions in both groups of rats were not statistically significant (Student's-t-test, two-tailed, with Bessel's correction)

N.D. = Not detectable by the sensitivity of the method (i.e. below 20 ng/tissue sample)

TABLE 7A

HMPG CONCENTRATIONS IN DIFFERENT BRAIN REGIONS 7 DAYS FOLLOWING COBALT-  
IMPLANTATION INTO THE RIGHT FRONTAL CORTEX OF PVG RATS

- values are expressed as ng/g wet tissue weight

S.D. = standard deviation

Sample	7 Days Post-Cobalt Implantation					7 Days After Operation in Sham Controls				
	RFC	LFC	RS	LS	OcC	RFC	LFC	RS	LS	OcC
1	120	105	-	99	-	86	95	82	90	85
2	102	99	121	132	88	55	68	79	71	65
3	95	98	55	98	92	93	80	74	79	89
4	-	118	98	98	51	85	83	91	80	51
5	83	115	95	88	97					
6	88	125	102	107	78					
Mean	98	110	94	104	81	80	82	82	80	73
+ S.D.	14	20	24	15	18	15	11	7	8	15

The differences in concentrations in respective brain regions of both groups of rats were not statistically significant (Student's-t-test, two-tailed, with Bessel's correction)

(-) = sample lost during the assay

TABLE 7B

HVA LEVELS IN THE STRIATUM 7 DAYS AFTER COBALT-IMPLANTATION INTO THE RIGHT FRONTAL CORTEX IN PVG RATS

- concentrations are expressed as ng/g wet tissue weight

S.D. - standard deviation

Sample	7 Days Post-Cobalt Implantation				7 Days After Operation in Sham Controls			
	RFC	LFC	RS	LS	RFC	LFC	RS	LS
1	N.D.	N.D.	472	437	N.D.	N.D.	608	582
2	"	"	444	494	"	"	702	667
3	"	"	534	604	"	"	558	562
4	"	"	639	741	"	"	599	658
5	"	"	615	551				
6	"	"	663	531				
Mean	N.D.	N.D.	561	560	N.D.	N.D.	615	617
+ S.D.	-	-	91	96	-	-	52	46
-								

The differences in concentrations between respective brain regions in both groups of rats were not statistically significant (Student's-t-test, two-tailed, with Bessel's correction)

N.D. = Not detectable by the sensitivity of the method (i.e. below 20 ng/tissue sample)



TABLE 8A

HMPG CONCENTRATIONS IN DIFFERENT BRAIN REGIONS 11 DAYS FOLLOWING COBALT-IMPLANTATION INTO THE RIGHT FRONTAL CORTEX OF PVG RATS

- values are expressed as ng/g wet tissue weight

S.D. - standard deviation

Sample	11 Days Post-Cobalt Implantation					11 Days After Operation in Sham Controls				
	RFC	LFC	RS	LS	OcC	RFC	LFC	RS	LS	OcC
1	75	94	98	110	54	77	87	100	90	85
2	-	99	105	111	76	93	85	88	88	78
3	92	115	60	-	76	72	74	84	73	89
4	82	77	115	92	72	74	79	78	72	48
5	60	96	97	103	70					
6	99	88	97	87	59					
Mean	82	95	95	101	68	79	81	88	81	75
+ S.D.	14	12	17	11	8	10	5	8	10	19

The differences in concentrations in respective brain regions of both groups of rats were not statistically significant (Student's-t-test, two-tailed, with Bessel's correction)

(-) = sample lost during the assay

TABLE 8B

HVA LEVELS IN DIFFERENT BRAIN REGIONS 11 DAYS AFTER COBALT-IMPLANTATION INTO THE RIGHT FRONTAL CORTEX IN PVG RATS

- concentrations are expressed as ng/g wet tissue weight

S.D. - standard deviation

Sample	11 Days Post-Cobalt Implantation				11 Days After Operation in Sham Controls			
	RFC	LFC	RS	LS	RFC	LFC	RS	LS
1	N.D.	N.D.	502	458	N.D.	N.D.	570	551
2	"	"	448	625	"	"	580	656
3	"	"	499	884	"	"	752	698
4	"	"	906	731	"	"	660	673
5	"	"	769	675				
6	"	"	606	581				
Mean	N.D.	N.D.	622	659	N.D.	N.D.	641	645
+ S.D.	-	-	164	132	-	-	73	56

The differences in concentrations between respective brain regions in both groups of rats were not statistically significant (Student's-t-test, two-tailed, with Bessel's correction)

N.D. = Not detectable by the sensitivity of the method (i.e. below 20 ng/tissue sample)

POST-MORTEM STABILITY OF HMPG AND HVA1. HMPG STABILITY IN POST-MORTEM BRAIN TISSUEA. Before freezing in liquid nitrogen

Exposure of the frontal cortex to different environmental conditions of time and temperature resulted in the following HMPG concentrations (ng/g tissue wet weight  $\pm$  S.D.) on subsequent assay (see Table 9 for details):-

## 1. Immediate homogenization and assay.

The mean concentration in this condition was 81 ng/g  $\pm$  9 S.D. (n = 5).

## 2. Immediate freezing and storage in liquid nitrogen.

The mean HMPG concentration in this condition was 88 ng/g  $\pm$  12 S.D. (n = 5).

## 3. Ice, for 30 min.

The mean HMPG concentration in this condition was 144 ng/g  $\pm$  23 S.D. (n = 5). This increase in concentration was significant when compared to conditions (1) and (2) above ( $P = 0.001 - 0.005$ ). and,

## 4. Decapitated head on the bench.

The mean HMPG concentration in frontal cortex tissue was 166 ng/g  $\pm$  39 S.D. (n = 5).

Again, the increase in concentration was significant when compared to conditions (1) and (2) above ( $P = 0.001 - 0.005$ ). Differences in concentration between condition (3) and (4) were



TABLE 9

HMPG CONCENTRATIONS IN FRONTAL CORTEX OF PVG RATS UNDER DIFFERENT CONDITIONS BEFORE FREEZING IN LIQUID NITROGEN

- values are expressed as ng/g wet tissue weight

Sample	Condition 1	Condition 2	Condition 3	Condition 4
	Immediate homogenisation	Immediate storage in liquid nitrogen	Ice (0°C) for 30 mins	Head on bench at room temperature (20°C) for 30 mins
1	84	95	122	121
2	76	89	122	223
3	91	86	125	138
4	69	103	128	148
5	85	66	173	198
Mean	81	88	144 *	166 *
+ S.D.	9	12	23	39

Statistical evaluation done with Student's t-test, two-tailed, with Bessel's correction

\* Significantly higher at P 0.001-0.005 when compared to condition 1 or 2

not significant. Moreover, delay and exposure to higher temperatures than solid CO<sub>2</sub> before freezing in liquid nitrogen resulted in increased variability of HMPG estimates in brain tissue as revealed in the increased standard deviation in conditions (3) and (4) when compared to (1) and (2).

#### B. After freezing in liquid nitrogen

Before homogenization, exposure of frontal cortex to different conditions yielded the following HMPG levels (ng/g wet tissue weight  $\pm$  S.D.). (For details, see Table 10):-

##### 1. Solid CO<sub>2</sub> for 5 min.

In this condition, the mean concentration of the metabolite was 86 ng/g  $\pm$  16 S.D. (n = 6).

##### 2. On ice, for 5 min.

The mean HMPG concentration was 163 ng/g  $\pm$  46 S.D. (n = 6). This increment in concentration was significant at P = 0.001 - 0.005 when compared to condition (1). Besides, the variability is also increased at this higher temperature compared to that due to (-75°C) of solid CO<sub>2</sub>.

and,

##### 3. Room temperature, for 5 min.

The mean HMPG concentration at this condition was 172 ng/g  $\pm$  48 S.D. (n = 6). Similarly, the increase in concentration was significant at P = 0.001 - 0.005, when compared to condition (1) above. Besides, the variability was also increased (S.D. = 48) when

TABLE 10

HMPG CONCENTRATIONS IN FRONTAL CORTEX OF PVG RATS UNDER DIFFERENT CONDITIONS AFTER FREEZING IN LIQUID NITROGEN

- values are expressed as ng/g wet tissue weight. Each value is the average of a duplicate estimate

Sample	Condition 1	Condition 2	Condition 3
	Solid CO <sub>2</sub> (-75°C) for 5 mins	Ice (0°C) for 5 mins	Bench (room temperature of 20°C) for 5 mins
1	95	165	227
2	105	227	186
3	84	220	239
4	83	122	125
5	62	119	140
6	86	123	117
Mean	86	163 *	172 *
+ S.D.	16	46	48

Statistical evaluation was done using the Student's-t-test with Bessel's correction

\* Significantly higher when compared to condition 1 at P 0.001-0.005

**TABLE 11****PRECISION OF DUPLICATE ESTIMATES OF HMPG CONCENTRATION AT SELECTED RANGES**

HMPG Concentration (ng/g wet tissue weight)	60 - 110	110 - 160	160 - 210	> 210
Total number of duplicates in a group	13	8	5	4
S.D. of difference between duplicates	7.4	14.5	15	19.7
Average % variability between all duplicates in a group	11.1%	10%	9.5%	11.6%

compared to that of solid CO<sub>2</sub> (S.D. = 16).

#### Precision of HMPG duplicate estimates

In the present study, and under the different conditions described above, there was a wide range of HMPG concentrations. The variance between duplicate estimates was found to increase in a direct proportion to the mean. In spite of the increased variance (S.D.) with higher concentrations, the average percentage variability or error was but little altered, indicating precision of duplicate HMPG estimates by the micromethod over a wider range of detectable concentrations of the metabolite.

Table 11 shows the standard deviation of the difference between such duplicate estimates calculated at intervals of 50 ng over the entire concentration range together with the % error between duplicates in each group.

## 2. HVA STABILITY IN POST-MORTEM BRAIN TISSUE

### A. Before freezing in liquid nitrogen

Exposure of the rat striatum to different environmental conditions of time and temperature resulted in the following HVA concentrations (mean ng/g tissue wet weight  $\pm$  S.D.):-

#### 1. Immediate homogenization and assay.

The mean concentration in this condition was 804 ng/g  $\pm$  61 S.D. (n = 5).

#### 2. Immediately to liquid nitrogen storage.

The mean concentration in this condition was 815 ng/g



TABLE 12

HVA CONCENTRATIONS IN RAT STRIATUM AT DIFFERENT CONDITIONS BEFORE FREEZING IN LIQUID NITROGEN

- all concentrations are expressed as ng/g wet tissue weight

Sample	Condition 1 Immediate homogenisation	Condition 2 Immediate (-195°C) freezing in liquid nitrogen	Condition 3 Ice (0°C) for 30 mins	Condition 4 Head on bench at room temperature (20°C) for 30 mins
1	731	720	729	910
2	843	763	754	776
3	768	838	804	686
4	888	868	1016	1154
5	792	885	1072	1135
Mean	804	815	875	932
+ S.D.	62	71	158	210

The differences between the 4 conditions were not statistically significant (Student's-t-test, two tailed, with Bessel's correction)

The variability was increased, however, with higher temperatures

$\pm 71$  S.D. ( $n = 5$ ).

3. Ice for 30 min.

The mean concentration was  $875 \text{ ng/g} \pm 158$  S.D. ( $n = 5$ ).

and,

4. Decapitated head on bench for 30 min.

The mean concentration was  $932 \pm 210$  S.D. ( $n = 5$ ).

(For details, see Table 12).

Although there was a trend to increasing concentration with delay and higher temperatures than solid  $\text{CO}_2$  ( $-75^\circ\text{C}$ ), there was no significant difference in striatal HVA concentrations between any of the conditions of treatment above. However, the variability was more marked at increasing temperature. The S.D. in conditions (3) and (4) were  $\pm 158 \text{ ng/g}$  and  $\pm 210 \text{ ng/g}$  respectively, in comparison to  $\pm 51$  and  $\pm 71$  of conditions (1) and (2) above, respectively.

B. After freezing in liquid nitrogen

Exposure of the rat striatum to different temperatures gave the following HVA levels (mean in  $\text{ng/g}$  wet tissue weight  $\pm$  S.D.).

For details, see Table 13.

1. On solid  $\text{CO}_2$  for 5 min.

The mean HVA concentration at this condition was  $821 \text{ ng/g}$

$\pm 78$  S.D. ( $n = 5$ ).

2. On ice for 5 min.

The mean concentration at this condition was  $906 \text{ ng/g} \pm 198$

S.D. ( $n = 5$ ).

TABLE 13

HVA CONCENTRATION IN RAT STRIATUM AT DIFFERENT CONDITIONS AFTER STORAGE  
IN LIQUID NITROGEN

- all concentrations are expressed as ng/g wet tissue weight

Sample	Condition 1	Condition 2	Condition 3
	Solid CO <sub>2</sub> (-75°C) for 5 mins	Ice (0°C) for 5 mins	Bench (room temperature of 20°C) for 5 mins
1	864	982	982
2	924	848	687
3	821	1174	996
4	720	632	592
5	778	893	958
Mean	821	906	843
S.D.	78	198	170

The differences between the 3 conditions were not statistically significant  
(Student's-t-test, two-tailed, with Bessel's correction)

The variability was increased, however, with higher temperatures

3. Room temperature for 5 min.

The mean HVA concentration at this condition was  
 $843 \text{ ng/g} \pm 170 \text{ S.D. (n = 5)}$ .

Again, the differences in HVA concentrations were not significant in comparison to condition (1) above. However, the variability was increased at higher temperatures. S.D. at  $0^{\circ}\text{C}$  and  $20^{\circ}\text{C}$  was  $\pm 198$  and  $\pm 170$  in comparison to  $\pm 78$  at  $-75^{\circ}\text{C}$ .

### Precision

An estimate of precision was obtained from consideration of the variance between duplicate estimates.

The variance between duplicate estimates was also found to increase in a direct proportion to the mean. This is illustrated in Table 14, which shows the standard deviation between such duplicate estimates calculated at intervals of 200 ng/g wet tissue weight over the entire range of concentrations.

In spite of the slightly increased variance between different concentration ranges, the percentage error or variability between duplicates in any range of concentration was but little affected, indicating precision of the method over a wide range of detectable concentrations of HVA.

TABLE 14

PRECISION OF DUPLICATE ESTIMATES OF HVA CONCENTRATION AT SELECTED RANGES

HVA Concentration (ng/g wet tissue weight)	600 - 800	800 - 1000	> 1000
Total number of duplicates in a group	13	12	5
S.D. of difference between duplicates	40.3	55.9	72.7
Average % variability between all duplicates in a group	5.9%	6.8%	7.5%

## DISCUSSION

HMPG and HVA concentrations were measured in various brain regions during the development of cobalt induced epilepsy in rats. The metabolite levels can be utilized in normal conditions as criteria reflecting noradrenaline and dopamine turnover of the catecholamine system, respectively.

### Assay method

A micromethod used by Ashcroft et al, (1975) was used for this assay. The method allows the estimation of HMPG and either HVA or 5-hydroxyindole acetic acid (5-HIAA) in brain tissue or CSF. The results of the present work confirmed the claims of the method in being useful, accurate and precise for measurement of both HMPG and HVA levels in small amounts of brain tissue.

For HMPG in brain, the method is used for tissues of 100 mg and less in weight, and the minimum amount of tissue required for fluorimetric estimation of HVA by the method was 15 mg of striatum and 100 mg of frontal cortex of the rat brain.

For HMPG estimation, the method employed the formation of the electron-capturing derivative, acetyl-TFA-HMPG, for its GLC estimation and was quite sensitive for measuring the small amount of

HMPG in small amounts of brain tissue. The method was quite sensitive for measuring the small amount of HMPG as that found in the rat brain. This is in contrast to the spectrofluorimetric method of Antun et al (1971) which has a sensitivity equivalent to 200 ng HMPG and is reproducible for micrograms HMPG/g brain tissue

weight which makes it unsuitable for the small amounts of HMPG found in the rat brain.

The micromethod showed a linear relation between the formation of the electron capturing derivative of HMPG and the various amounts of HMPG in pure solution or extracted after being added to brain homogenates. The percentage recovery of the method (77.4%) was more than that reported for the GLC method employing the formation of heptafluorobutyl HMPG (64%) (Sharman, 1969). The sensitivity of the method was equivalent to 2 ng HMPG per tissue sample and its specificity by paper chromatography was shown by Walter (1973).

For HVA estimation, the micromethod employed the formation of HVA fluorophore, and showed a linear relation between fluorophore formation and the different amounts of HVA extracted from aqueous solutions or added to brain homogenates. The percentage recovery of added HVA by the method was very high (96%). Thus, for subsequent HVA assays in brain extracts, no correction for tissue recovery was essential, and this is one of the advantages of the method. In contrast, correction of HMPG concentrations for tissue recoveries was always required.

The sensitivity of the method for HVA estimates was equivalent to that of 20 ng HVA per tissue samples. This makes it more suitable for tissues rich in dopamine such as the striatum. However, the fluorimetric estimation of HVA by the micromethod might be useful for measuring dopamine turnover in tissues of lower dopamine content such as the frontal cortex if an adequate amount of tissue is used, or if a marked increase in dopamine turnover or accumulation of its metabolite,

HVA, is anticipated. The HVA estimation by this method has a high degree of specificity (Ashcroft et al, 1968), as was shown by paper chromatography.

#### The epileptic changes following cobalt application

Following the implantation of cobalt into the right frontal cortex of the rat, a primary epileptic focus developed in the nervous tissue around the implant and a secondary focus is formed in the corresponding area of the contralateral cortex, as measured in the ECoG. Spike activity appeared in both foci in 3 days and increased in frequency reaching a maximum in 8 - 12 days after the implantation. This same pattern was reported by other workers (Dow et al, 1972, Emson & Joseph, 1975). The frequency of spiking in the secondary focus was almost always greater than in the primary focus.

It was difficult to separate the causative epileptiform mechanisms, since neuronal damage alone in the cobalt-implanted area and in the secondary focus might be sufficient to initiate epileptic activity, similar to cases of post-traumatic epilepsy. Some workers claimed that the cobalt ion is the critical factor (Dow et al, 1962, Willimore et al, 1975). Willimore et al (1975), employing a 100 mMol cobalt chloride solution in an iontophoretic study, demonstrated that the cobalt ion did possess a potent epileptogenic effect. This was observed after micro-iontophoretic transfer of at least 100  $\mu$ g of ionic cobalt onto the pial surface of the sensorimotor cortex. These workers reported that with this method, no histological evidence of cell damage was found and that although about 75% of the cobalt was found in layer



1, the neurons and their processes were intensely stained in layers II and III of the cortex. Thus, epileptiform discharge originated from neuronal aggregates that have selective affinity for ionic cobalt at the focal site in the upper layers of sensorimotor cortex.

Some workers pointed out that the formation of a meningeal cicatrix also contribute to the development of epileptic activity (Payan, 1971, Fischer et al, 1968).

Moreover, glass implants, which could cause some meningeal damage and gliosis, did not initiate epileptic activity (Dow et al, 1972). However, it seemed that both factors, the general cell damage and cobalt toxicity, contributed towards the epileptic phenomena. The importance of contribution of each factor varied at different stages of the epileptic process. The fact that cobalt ion was found in primary and secondary cortical foci as well as striatum and occipital cortex on both sides as early as 6 days after implant and was still found in considerable concentration after 3 weeks (Clayton, 1976), suggests that both factors are operating during the development of spike activity in cortical neurons. However, the epileptic activity after 10 weeks of cobalt implantation, when cobalt ion was cleared from all brain areas except the primary focus (Clayton & Emson, 1975), probably reflects the cell damage which is largely permanent. These workers used atomic absorption spectrophotometry (which allows unequivocal estimation of cobalt ions) and heavy metal histochemistry in showing that cobalt ion spread widely from the site of the implant. Cobalt ions might spread by diffusion or by being transported away from the original lesion to establish a gradient of cobalt throughout the brain. Spread of ionic by the bloodstream might also be possible. However, the amount of cobalt

spreading by diffusion is unlikely to be so extensive outside the primary lesions. Systemic spread of cobalt ions from the side of implant by bloodstream would have lead to its deposition in other organs, particularly those of the reticulo-endothelial system such as the liver and spleen, which is not the case. Thus, it is probable that the majority of cobalt was spread to the rest of the brain by axonal transport. In agreement with this suggestion, the levels of cobalt ion in the contralateral caudate were found to follow closely those in the overlying cortex with which it had definite fiber connections (Clayton & Emson, 1976). Furthermore, it is well known that cobalt salts could be used to trace axonal pathways (Pitman, Tweedle & Cohen, 1972).

#### Catecholamine turnover in cobalt-implanted rats

Tyrosine hydroxylase activity was reported to be the rate-limiting step of catecholamine biosynthesis, and the most specific marker of the functional integrity of catecholaminergic fibers (Spector et al, 1965, Levitt et al, 1965). It is also well known that monoamine oxidase and catechol-O-methyltransferase are the main degrading enzymes. It was reported that in this time period, tyrosine hydroxylase activity was reduced in primary and secondary foci reaching 10% of its value at 6 days, and was still down at 12 days in both primary and secondary cortical foci, but had partially recovered at 30 days after cobalt implantation reaching to 30% of its activity (Clayton & Emson, 1975). In the striatum, a similar reduction in enzyme activity was observed (McQueen & Emson, 1975, unpublished data). Reduction in the activity of monoamine oxidase and catechol-O-methyl transferase reaching to 20% and 40% of their control value paralleled these changes (Clayton & Emson, 1975). However, noradrenaline concentration was found to be normal in cortical foci, using

biochemical (Clayton, 1975), and fluorescence methods (Emson & Bjorklund, 1974 - unpublished data) of assay. Similarly, dopamine and noradrenaline concentrations in striatum did not significantly differ from those of the reported control values. (See Section II : Results : 6-OHDA experiment in cobalt implanted rats).

These changes might imply that the enzyme changes were a reflection of local cortical events, induced by cobalt, rather than a general reaction of the catecholamine system in the brain. The finding of normal tyrosine hydroxylase activity in the locus coeruleus, where most noradrenergic neurons originate (Ungerstedt, 1971, Dahlstrom & Fuxe, 1965), and in the midbrain in cobalt-implanted rats (Clayton, 1975), tended to support this hypothesis. Thus, it seemed that the local catecholamine turnover was reduced at sites where the cobalt ion and its accompanying cellular change were found, but normal concentration of noradrenaline in cortex and both catecholamines in the striatum was maintained by compensatory enzymatic change.

#### HMPG and HVA levels in cobalt-implanted rats

During the different stages of development of epileptic activity in the primary and secondary foci in cobalt-implanted rats, HMPG and HVA levels were not significantly different in comparison to levels in control rats. However, HMPG levels tended to be higher at 7 and 8 days after cobalt implantation, when epileptic activity was very marked, in comparison to those seen earlier after 3 days when epileptic activity was developing and also in comparison to those levels of the metabolite seen at 11 days following cobalt application when epileptic activity was still prominent. HVA levels in striatum remained generally unchanged during these time

periods although there was a non-significant drop at 7 and 8 days after cobalt application.

#### Possible mechanisms

Cobalt implantation in the brain caused large areas of necrosis. This was marked in the primary focus. Histological evidence of cellular degeneration were also reported in the homologous contralateral cortex in secondary focus, the striatum on both sides as well as in some regions of the thalamus. It was possible that the enzyme changes observed might not be specific but due to neuronal degeneration.

Various parameters in the catecholamine nerve terminals involving synthesis and breakdown of the neurotransmitter, appeared to be sensitive to toxic effect of cobalt ion, and this was evident in the primary focus. Thus, one had to assume a fairly rapid transport of ionic cobalt to the contralateral side to account for the parallel enzymatic changes in the secondary focus. Cobalt ion was demonstrated in the contralateral cortex as early as 6 days after implantation (Clayton, 1976). Thus, the cobalt ion in the cortex, concentrated in the region of catecholamine nerve terminals might effect a permanent alteration in enzyme function whether they are synthesizing, catabolising or transport-associated enzymes. Cobalt ion might produce its toxicity by damaging the enzyme function, possibly by alteration of structure or by interfering or inhibiting the action of enzyme co-factors (Pitman et al, 1971, Payan, 1971, Dow et al, 1962). Furthermore, cellular changes might potentiate the effects of the enzyme changes induced by cobalt.

The finding of unaltered concentration of HMPG in the face of

reduced catecholamine synthetic activity might be explained by increased noradrenaline catabolism or by a reduction in HMPG transport from the brain. The presence of normal concentrations of noradrenaline as well as reduced monoamine oxidase and catechol-O-methyl transferase activity in epileptic foci tended to exclude the former possibility. However, this would have resulted in reduced HMPG levels at these sites. Thus, it seemed reasonable to suggest inhibition of HMPG transport in the presence of reduced catabolism of noradrenaline, contributing to a normal or tendency to increased concentration of the metabolite in cortical foci and striatum. This block in HMPG transport mechanism could be attributed to the toxic effect of cobalt on the enzyme system, as described above, potentiated by the associated cellular degeneration and membrane damage.

The assay method as used in the present work measured the total HMPG and did not distinguish free from conjugated HMPG. However, in the rat brain, about 90% of total HMPG occur as HMPG-SO<sub>4</sub>.

Free HMPG is thought to be readily diffusible. Some workers believed that no transport system existed for HMPG since it could diffuse so easily out of the brain into CSF or blood that no need for transport is demanded (Korf et al, 1971). This theory was supported by the finding of low regional variation of HMPG in the brain. In the rat brain, only a two-fold maximal regional difference for total HMPG was found, the highest being in the brain stem and lowest in the cerebellum (Yates - personal communication). Similarly, a maximal four-fold variance in HMPG was found in the dog brain (Ashcroft et al, 1975), and similar results were reported for the human brain (MacKay et al, 1978). This is in contrast to HVA which showed up to a ten-fold regional variation

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and also in contrast to the pattern of localization of monoamine neurotransmitters.

HMPG removal from the brain differed from HVA in being relatively insensitive to probenecid. Barany (1975) established the existence of two distinct transport mechanisms in the rat choroid plexus. In addition to the classical renal-like system, another system could be demonstrated which was less sensitive to blocking by probenecid. It was possible for HMPG to be a candidate for the latter system. Eccleston & Ritchie (1973) have shown that HMPG was readily esterified in the rat brain, *in vivo*, and that there was evidence of HMPG-SO<sub>4</sub> transport mechanism in the rat brain (Miner & Heston, 1972, Mrsulja et al, 1975), which resembles that of HVA and 5-hydroxyindolacetic acid in being inhibited by probenecid and by ischemia (Meek & Neff, 1972, Miner & Heston, 1972, Mrsulja et al, 1975).

Thus, impairment of transport of HMPG-SO<sub>4</sub> out of the brain into CSF or blood, as induced by cobalt ion toxicity and cellular degeneration would lead to its retention in brain tissue HMPG.

In the presence of reduced noradrenaline catabolism, HMPG retention, at the site of primary and secondary cortical foci as well as in striatum on both sides, could build up to normal or even a tendency to a higher concentration of the metabolite. This would give a false impression of normal or increased turnover of noradrenaline at these sites in the presence of the opposite effect. However, impaired HMPG-SO<sub>4</sub> transport acting alone would lead to progressive accumulation of the metabolite in brain tissue, which is not the case. HMPG levels were higher in cortical foci and striatum at 7 and 8 days when compared to those at 3



days following cobalt implantation, but these levels of the metabolites were lower after 11 days in comparison to those at 7 or 8 days after cobalt application. However, at all these time intervals, the levels were not significantly different from control. Thus there might be a factor which limited HMPG accumulation and this could be reduced by HMPG-SO<sub>4</sub> formation inside the neuron.

It is also possible that cobalt ion could damage the intracellular sulphatase enzymes systems, thus impairing the sulphate conjugation of free HMPG which normally resulted in formation of HMPG-SO<sub>4</sub>. This would render a higher proportion of HMPG in the free or diffusible form in the damaged areas. Since free HMPG readily passes into blood or CSF, this would prevent or limit the accumulation of higher levels of the metabolites which might result from impaired transport of its conjugated form.

It could be possible that the enzyme-mediated transport of HMPG-SO<sub>4</sub> is impaired earlier than the intracellular sulphatase enzyme system which normally conjugated the free form of the metabolite. This could mean that the non-significantly higher levels of the metabolite at 7 and 8 days after cobalt application was due to mild HMPG-SO<sub>4</sub> retention. At 11 days after cobalt-implantation, however, it was possible that a higher proportion of HMPG existed in the free diffusible form in both epileptic foci and striatum, hence, the tendency to lower values at these sites in comparison to those seen at 7 or 8 days following cobalt application. However, the net result of these changes at these different times was that the metabolite levels ceased to reflect the locally reduced noradrenaline turnover in spite of the normal concentration of noradrenaline.



Similar mechanisms could operate for HVA levels in the striatum where cellular degeneration and ionic cobalt were reported following cobalt application to frontal cortex.

Tyrosine hydroxylase activity was also associated with reduced activity of monoamine oxidase and catechol-O-methyl transferase in the striatum. Similar to noradrenaline, the concentration of dopamine in the striatum was maintained in the presence of reduced synthesis, by a compensatory reduction in its catabolism. This would have resulted in reduced levels of HVA in the striatum. However, the present results showed that the levels of HVA were maintained in comparison to control value. The degenerative cellular changes in addition to the cobalt-induced enzyme toxicity could impair the active transport of the reduced amount of HVA formed and this could build up to normal or even a tendency to higher levels. However, there was no evidence of increased HVA concentrations with time. Sharman (1967) showed that a small part of HVA, which corresponded to the small intraneuronally formed pool, was excreted normally by diffusion into the blood independently of and in parallel to the actively excreted part. It might be possible to suggest that the formation of the intraneuronal diffusible pool of HVA is proportionately increased to limit possible accumulation of HVA that might result from progressive mild impairment of active transport with time. This might explain the absence of significant changes in the metabolite level in the striatum of cobalt-implanted rats in the presence of reduced dopamine catabolism.

The absence of detectable amounts of HVA in the primary and secondary foci in the frontal cortex might reflect the lack of dopaminergic terminals in these areas. Alternatively, the impairment of HVA

transport in these areas of cellular degeneration, might not be sufficient to render it detectable by the sensitivity of the method which is 20 ng HVA per tissue sample.

These findings implied that estimation of the concentration of the major amine metabolite is not a good index of amine turnover in the presence of cellular degeneration and necrosis in comparison to normal functioning tissue. This was due to the accompanying enzymatic damage with possible alterations in metabolite active transport mechanism or even changes in metabolite formation.

Thus, these changes in the metabolite levels might be misinterpreted to indicate normal or even increased amine turnover in the presence of the opposite effect.

In this context, it might be worth attempting to explain the contradictory finding of low tyrosine hydroxylase activity in the presence of normal noradrenaline concentrations. The following explanations might all contribute in part to these findings:-

- (a) There might be some compensatory increase in the noradrenaline concentration in the remaining undamaged neurons.
- (b) It is generally accepted that the levels of the transmitter synthesizing enzymes were in excess to what is required for normal nerve function, but tyrosine hydroxylase activity was severely reduced to about 10% of its activity in both cortex and striatum after cobalt implantation. Thus, this explanation would seem unlikely.

- (c) The activity of tyrosine hydroxylase might be influenced by local events in vivo which could impair its effectiveness as a rate-limiting enzyme in the biosynthesis of catecholamines.

MacKay (1974) showed that in the sympathetic ganglion preparation, no linear relation was found between tyrosine hydroxylase activity and noradrenaline concentrations. Other workers have reported a dissociation between the enzyme activity in vivo and in vitro, and even questioned its role as the rate-limiting enzyme. It was well known that the enzyme depended on its activity mainly on the availability of the reduced folate co-factor (Turner et al, 1974) which was made available by the enzyme dihydrofolate reductase. It was likely that local cortical necrosis and devascularization and/or the toxic effect of cobalt ion would interfere with the local supply of the reduced co-factor or the affinity of the enzyme for it, thus reducing its activity in a dramatic manner.

The enzymes L-aromatic amino acid decarboxylase and dopamine  $\beta$ -hydroxylase were also involved in the biosynthesis of noradrenaline. However, the decarboxylase enzyme was reported to be reduced in cortical foci in cobalt-implanted rats (Emson & Joseph, 1975). Thus, it might be possible that increased dopamine  $\beta$ -hydroxylase activity would contribute to the normal levels of noradrenaline in noradrenergic terminals in presence of reduced tyrosine hydroxylase activity, provided that it was not damaged by the pathological changes. It has already been suggested that when significant levels of dopamine accumulated in the nerve endings, then dopamine  $\beta$ -hydroxylase or the intravesicular uptake of dopamine became rate-limiting in noradrenaline biosynthesis (Landsberg et al, 1969). However, it is well known that alpha-methyltyrosine, which inhibits

tyrosine hydroxylase, causes catecholamine depletion in vivo under normal conditions. Thus, it seemed that the dopamine hydroxylase did not compensate enough for the reduced tyrosine hydroxylase activity under these circumstances and consequently the concentration of noradrenaline was reduced.

- (d) Reduced catabolism of noradrenaline by monoamine oxidase and catechol-O-methyl transferase might contribute to the normal concentration of the reduced amount of noradrenaline synthesized via tyrosine hydroxylation.

Monoamine oxidase and catechol-O-methyl transferase are found mainly related to catecholaminergic neurons, but also in non-catecholaminergic neurons and glia (Robinson, 1967, Katz et al, 1969). These enzymes might be specifically inhibited by cobalt ion or extensive cellular damage which would not agree with the findings of normal fluorescence of noradrenergic terminal in cortical foci (Emson & Bjurklund - unpublished data), or with extent of degeneration and necrosis found histologically. It was also possible that cobalt ion might have first affected a reduction in tyrosine hydroxylase activity while the changes in monoamine oxidase and catechol-O-methyl transferase might be merely a compensatory phenomenon.

From all the above findings one could conclude that cobalt implantation and its accompanying tissue necrosis reduced the local catecholamine turnover. However, the normal concentrations of catecholamines were maintained by compensatory enzymatic mechanism. HMPG and HVA concentrations, however, did not reflect the turnover of noradrenaline and dopamine, respectively, due to the degenerative and

enzymatic changes which could impair the formation or active transport of these metabolites from their sites of formation in the brain to CSF or blood stream. Furthermore, the presence of neuronal degeneration and its local denervation supersensitivity in the epileptic foci might render the remaining cortical neurons more hyper-excitabile or responsive to the normal concentrations of noradrenaline present, thus contributing to the resulting process of epileptogenicity.

Using a modification of the cobalt implantation method in immunologically-suppressed animals, some workers showed that cobalt ion could produce focal epileptic changes without possible alterations in catecholamine metabolism and before the onset of substantial cortical and sub-cortical necrosis with its associated enzyme changes (Altamura et al, 1978). With this method, the development of epileptic manifestations, however, was delayed and reached a maximum after 15 days, while tissue necrosis both cortical and sub-cortical, was less marked and reached a peak later after 20 days.

This could mean that the biochemical findings in catecholamine metabolism already described in the present and previous work (Clayton & Emson, 1975, Clayton, 1976), were more due to the associated tissue degeneration and necrosis than to the toxic effect of cobalt ion on enzyme systems associated with catecholaminergic neurotransmission. However, the earlier onset of cellular degeneration in cortical foci with its possible local denervation supersensitivity facilitated the development of epileptic changes. The normal concentration of noradrenaline acting on the locally supersensitive post-synaptic receptors could have contributed to the neuronal hyperexcitability at these early stages.

A possible mechanism by which the cobalt ion could initiate epileptic changes, without producing necrotic changes, might be by affecting active ion transport through the neuronal membranes. Glial cells, particularly astrocytes, could act as buffers to protect neurons against an accumulation of extracellular potassium ion. It might be that cobalt ion induced a significant modification in oxidative metabolism of astrocytes characterized by increased dehydrogenase activity, apart from gliosis, with consequent focal epileptic changes. Such activated astrocytes have been found in focal epilepsy in man, and also in cobalt-induced epilepsy in the rat where they appeared before the first electrical phenomena of epilepsy (Brotchi et al, 1977).

#### Post-mortem stability of HMPG and HVA

In the time period following decapitation, the present work showed that exposure of brain tissue to increasing temperatures of 0°C (on ice) or 20°C (head on bench) for a period of 30 min before freezing and storage in liquid nitrogen, resulted in a significantly higher and more variable HMPG concentration on assay when compared to those found in brain tissue that had been homogenized immediately after decapitation or frozen in liquid nitrogen before being homogenized.

Similarly, in the time period following freezing the tissue in liquid nitrogen, HMPG concentration in frontal cortex tended to be higher and more variable on exposing the tissues to higher temperatures of 0°C (ice) or 20°C (room temperature) for 5 min when compared to that in tissues homogenized while being kept frozen on solid CO<sub>2</sub> for a similar period of time.

These observations might be explained by facilitation of the enzymatic catabolism of noradrenaline occurring with delayed homogenization on higher temperatures either before or after freezing the tissue in liquid nitrogen or solid CO<sub>2</sub>. This implied that delayed freezing of brain tissue allowed the catabolising enzymes, monoamine oxidase and catechol-O-methyl transferase to act, which effect was potentiated by higher temperature. This increased catabolism of noradrenaline (and dopamine) did not occur when the tissue was immediately frozen or homogenized after decapitation. Furthermore, HMPG and HVA concentrations did not differ significantly between tissues homogenized immediately or those stored frozen.

Furthermore, it implied that freezing inhibited the activity but maintained the stability of the catabolising enzymes whose activity was also enhanced by thawing at higher temperatures of ice and the room.

Other workers have also demonstrated the post-mortem stability of monoamine oxidase and catechol-O-methyl transferase in the rat as well as the human brain (Ganrot et al, 1962, Vogel et al, 1969, Mackay et al, 1978, Hakanson & Owman, 1966). This implied that freezing the tissues inhibited the activity of the catabolising enzymes, thus maintained brain dopamine and noradrenaline, respectively. This was supported by the finding of Walter (1973) that storage of brain tissue for 3 months in liquid nitrogen depressed the activity of the catabolising enzymes and did not alter HMPG levels on subsequent assay.

These results also showed that HMPG and HVA levels in tissue immediately homogenized after death or kept frozen immediately before



being homogenized without thawing, reflected the turnover of their parent catecholamines, noradrenaline and dopamine, respectively at the time of death. Furthermore, it showed that this method of tissue handling was the best method in determining the concentration of major catecholamine metabolites in reflecting the turnover of their parent amines at or just before the time of death.

The more rapid thawing of the brain tissue at higher temperature with its accompanying membrane lysis might result in increased release of dopamine and noradrenaline with their consequent enhanced enzymatic degradation, thus contributing to the significantly higher and more variable concentration of their metabolites. However, studying the post-mortem alterations of HVA in the rat striatum showed that it was more stable than HMPG in the altered conditions of time and temperature described above before and after freezing the tissues in liquid nitrogen. Noradrenaline and dopamine were known to be prone to a substantial post-mortem change (McGeer & McGeer, 1962, Grabartis et al, 1966, Vogel et al, 1969, Carlsson et al, 1974). However, the dopamine metabolite, HVA seemed to have a much greater post-mortem stability (Hornykiewicz et al, 1968). The present work showed that alteration in time and temperature did not significantly alter HVA concentration in striatum in comparison to that which was either immediately homogenized or frozen before being homogenized, confirmed this stability and suggested that HVA concentration at these conditions still reflected the dopamine content at the time of death. However, the increased variability of HVA content in brain tissue at higher temperature and with delayed homogenization either before or after freezing precluded using HVA level under these conditions as indicating dopamine turnover or just before the time of



sacrifice.

Some workers found that the noradrenaline content of rat brains homogenized at increasing time intervals (ranging from 30 min to 4 hrs) at room temperature were significantly lower than that in control brain immediately homogenized after death. This loss of noradrenaline did not occur in brains that were frozen immediately and homogenized without thawing, indicating that instant freezing generally depressed the activity of the catabolizing enzymes and protected brain noradrenaline (Grabartis et al, 1966). Moreover, brain dopamine and noradrenaline were reported to be significantly reduced in the brain of rats exposed at 4°C and 16°C for 16 hrs while monoamine oxidase and catechol-O-methyl transferase activities showed no significant alterations (Vogel et al, 1969). These changes occurred in spite of the fact that the tissue showed a considerable decomposition. Furthermore, impairment of binding and storage of dopamine and noradrenaline might also contribute to reductions in their levels with exposure of brain tissue to increasing temperature after death. Maynert & Kuriyama (1964) observed that tissue slices released dopamine and noradrenaline much faster than 5-HT during incubation, thus indicating that the concentration 5-HT is better maintained than the other monoamines in post-mortem preparations (Vogel, et al, 1969, Mackay et al, 1978).

It might be worth describing briefly the catabolism of dopamine and noradrenaline under the different conditions above.

The breakdown of dopamine by monoamine oxidase yields DOPAC which is methylated to HVA. Moreover, the O-methylation of dopamine by catechol-O-methyl transferase yields 3-methoxytyramine which is

deaminated into HVA by monoamine oxidase.

In contrast, the O-methylation of noradrenaline produces normetanephrine which is deaminated into HMPG by monoamine oxidase. Moreover, the deamination of noradrenaline yields DHMP which is O-methylated into HMPG.

The enzyme monoamine oxidase requires oxygen and its optimum activity occurs at 37°C. Catechol-O-methyl transferase depended on its activity on co-factors which are present in surplus in the tissues and thus the time between death and freezing of tissue or homogenization is critical before these co-factors are utilized.

It is well known that oxidative deamination, which requires oxygen stops after death while the tissue is inside the body, and any residual monoamine oxidase activity was due to the low oxygen content in the tissue at that time. This explained the enzyme action while the decapitated head in the experiment was left on the bench for 30 min before dissecting the brain tissue out. However, in experiments in which the brain tissue was put on ice or the bench, the atmospheric oxygen, in addition, contributed to the enzyme action. Exposure of brain tissue to 0°C (on ice) resulted in elevation of HMPG content while HVA concentration was unaltered. Thus, it seemed that monoamine oxidase had more affinity under these conditions for normetanephrine than to 3-methoxytyramine, thus contributing to higher concentrations of HMPG. However, the concentrations of DOPAC, formed from oxidative deamination of dopamine was found to increase under these conditions (Nicolaou - unpublished data). Thus, it also seemed that catechol-O-methyl transferase had more affinity for DHMP than DOPAC under these conditions in

contributing to the higher values of HMPG. The fact that these changes occurred within 5 min of exposure of brain tissue to ice (at 0°C) emphasized the importance of keeping the tissues frozen on solid CO<sub>2</sub> before homogenization in the HMPG assay. The higher levels of HMPG on putting the tissue on the bench (20°C) indicated the enhancing effect of temperature on the enzyme changes mentioned above.

However, in conditions where the decapitated head (with brain still inside) was left on the bench, the catabolism of dopamine and noradrenaline was mainly by COMT, and the activity of monoamine oxidase depended on the tissue oxygen at time of death. The increased HMPG content and normal HVA levels in this condition could also be explained by the mechanisms already described above, even under the adverse conditions of low oxygen tension for the action of monoamine oxidase. Moreover it has been shown that rapid accumulation of 3-methoxytyramine occurred in rat brain during the first hour after death at 37°C after decapitation, and this accumulation was enhanced by treatment with pargyline, a monoamine oxidase inhibitor and was less rapid at lower temperature. The post-mortem loss of dopamine from the brain corresponded to the formation of 3-methoxytyramine in pargyline-treated rats, but exceeded this formation in the non-treated rats (Carlsson et al, 1974) suggesting some deaminating function by monoamine oxidase, possibly from the residual dissolved tissue oxygen.

Thus, it might be possible that post-mortem alterations of time and temperature contributed to high levels of HMPG previously reported in cobalt-implanted rats. However, these changes are unlikely to alter HVA concentration which thus seemed to have better post-mortem stability.

The absence of significant changes in HVA levels in striatum at different times following cobalt implantation contrasted with the reduced levels of the metabolites observed before (McQueen & Emson - unpublished data). This could be explained by animal variation in the different grades of degeneration in the striatum with its consequent variable effects on active HVA transport as well as other compensatory mechanisms in the presence of reduced tyrosine hydroxylase activity in this region.

## SECTION 2

DOPAMINE AGONISTS AND  
COBALT-INDUCED EPILEPSY.

INTRODUCTIONA. DOPAMINE AS A NEUROTRANSMITTER

Dopamine (DA), like noradrenaline (NA) and adrenaline, is a catecholamine, possessing a 3,4-dihydroxy (catechol) aromatic ring and an amino group. These three biologically active amines are synthesized in series from l-tyrosine via L-DOPA. Dopamine was originally considered as a precursor, but by the 1950's was recognised to be pharmacologically active itself (Blaschko, 1973). Early evidence for the transmitter role of dopamine was derived from failure of noradrenaline and 5-HT to reverse some of the effects of the monoamine depleting drug, reserpine, and the demonstration of very high concentrations of dopamine in brain areas like the striatum which had low amounts of noradrenaline (Carlsson, 1959). Dopamine was also found to fulfil the criteria of neurotransmitter, as suggested by McLennan (1963). These included the presence of enzymes necessary for the synthesis and metabolism of the transmitter and the release after nerve stimulation. Moreover, local application of dopamine simulated nerve stimulation (Horneykiewicz, 1973).

Investigation of dopamine mechanisms had been greatly helped by the recognition of dopamine receptors in the central nervous system and periphery. Activation of these receptors by dopamine was accompanied by an increase in cyclic AMP via a dopamine-sensitive adenylyl cyclase (Greengard & Kebabian, 1974). Some drugs, like apomorphine, piribedil and bromocryptine, mimicked the stimulation of the effector tissue. Conversely, dopamine-receptor antagonists like chlorpromazine, haloperidol and pimozide, blocked the action of these agonists. The

dopamine agonist and antagonist properties of these drugs had been confirmed in several models in vivo (Ungerstedt, 1971, Goldberg, 1972).

## B. FUNCTIONS OF DOPAMINERGIC PATHWAYS

Fig. 12 shows the principle dopamine pathways in rat brain. Dopamine appears to produce mainly inhibitory (hyper-polarizing) responses at its receptors. In some parts of the brain, its effects are much more powerful than those of noradrenaline. The nigro-striatal pathway is primarily concerned with motor function, and the infundibular system with the regulation of hypothalamic releasing-factors (Vogt, 1973). Although dopamine mechanisms have been implicated in mood and cognitive functions, the evidence was less direct. Substantial evidence of a disorder of dopamine pathways in psychosis is derived from the observation that antipsychotic and neuroleptic activity correlated with dopamine receptor antagonist properties over a wide range of drugs with different structure (Matthysse, 1973, Snyder et al, 1974). In addition, amphetamines, which release endogenous dopamine, might provoke hallucination and psychosis (Snyder et al, 1974), as did L-DOPA and dopamine agonists (Calne et al, 1974). It is too simple to suggest that schizophrenia is a disease of dopamine overactivity in the limbic system (Crow, Johnstone, Deakin & Longden, 1976). However, it is possible that functional dopamine overactivity might contribute to the clinical features of psychosis. Animal experiments implicated dopamine in many other behaviour patterns including stereotypy (Costall & Naylor, 1975), eating, drinking and autonomic control (Breese, Cooper and Smith, 1974).

The relationship of dopamine system to seizure susceptibility is still obscure. Stimulation of the caudate nucleus was reported to

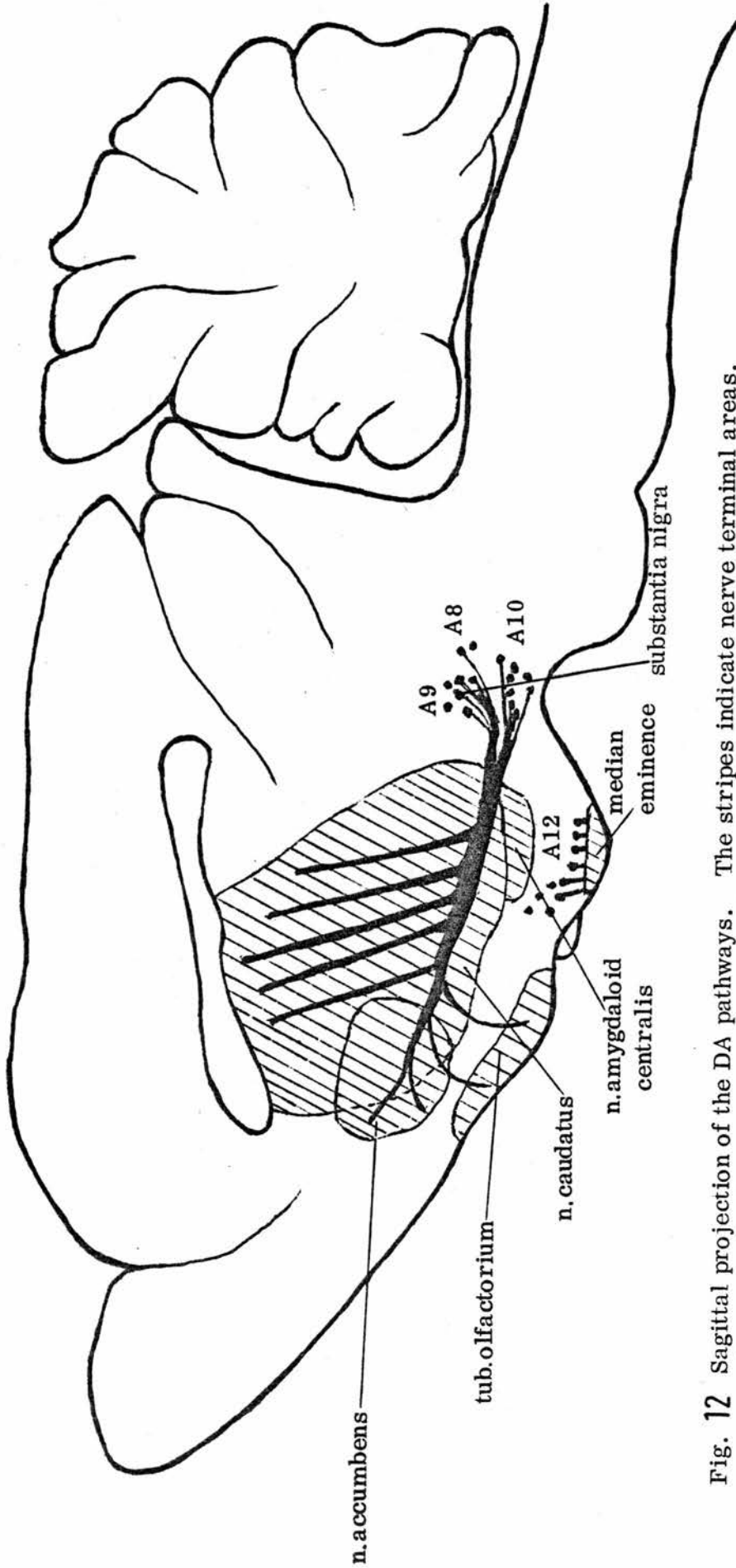


Fig. 12 Sagittal projection of the DA pathways. The stripes indicate nerve terminal areas. Shown are the long nigrostriatal fibers originating in the substantia nigra (Cell bodies A8 and A9), the long mesolimbic pathways originating from dopaminergic cell bodies surrounding the interpeduncular nucleus (A10) and the short tuberoinfundibular dopaminergic neurone with cell bodies in the arcuate nucleus (A12) in the hypothalamus.



produce arousal (Hensen et al, 1961), and to inhibit seizure activity in other brain structures (Umbach, 1959, La Grutta, Amato and Avellone, 1972). Moreover, abnormally high concentrations of dopamine in the brain were associated with arousal (Mantegazzini & Glaser, 1960), or with irritability and increased motor activity (See Vogt, 1973).

#### C. DOPAMINE METABOLISM AND CLINICAL EPILEPSY

In general, central catecholamine metabolism in man could be followed indirectly by findings in the blood, urine and CSF. Shaywitz (1969) and other workers (Bartolini & Hornykiewicz, 1967, Papeschi et al, 1972), found a reduction of about 50% in HVA concentration in the CSF of epileptic children. A similar reduction was also found in the CSF HVA levels of a group of untreated epileptic patients (Shaywitz, Cohen and Bowers, 1973), suggesting that low HVA concentrations in the CSF primarily reflected the disease process rather than being the effect of treatment. However, other workers reported no change in HVA concentration in CSF samples taken from patients with grand mal and temporal lobe epilepsy (Garelis & Sourkes, 1974), while others reported that CSF levels of HVA in unmedicated patients as close to control values (Chadwick, Jenner and Reynolds, 1975). These workers also reported a correlation between HVA levels in CSF and plasma anti-convulsant concentrations and suggested that various anti-convulsants raised CSF HVA levels and that HVA elevation was a manifestation of anti-convulsant effectiveness.

In these cases L-DOPA treatment was effective in suppressing epileptic fits (Bernheimer et al, 1966, Bartolini & Hornykiewicz, 1967). Similar results have been reached with L-DOPA and decarboxylase inhibitor

RO-4-4602 (Birkmayer, 1974). These data might suggest that measurement of CSF HVA levels might have some diagnostic value in some epileptic patients.

D. ALTERED MONOAMINE METABOLISM IN EXPERIMENTAL EPILEPSY

It has been shown that in audiogenic seizure, the seizure threshold in genetically susceptible mice varied with age, and this was correlated with age-induced changes in brain amine levels (Schlessinger et al, 1959). Some workers found that noradrenaline and 5-HT levels were 40% and 50% respectively, of their control values in non-sensitive mice at the time of maximal susceptibility to seizures. Moreover, at later times, when mice were no longer audiosensitive, their brain amine levels closely returned to normal values. Similarly, it was suggested that a similar change in noradrenaline occurred in rubidium chloride-treated audio-sensitive mice (Alexander & Meltzer, 1975). Paradoxically, however, it was then reported that rubidium increased noradrenaline turnover in rat brain (Stolk et al, 1970). In cobalt-induced epilepsy in rats, some workers failed to find changes in either noradrenaline, dopamine or 5-HT levels in whole brain or a change in noradrenaline or dopamine turnover (Colosanti & Craig, 1970). Furthermore, noradrenaline levels in cortical foci was found to be unaltered in comparison to control unoperated rats (Clayton, 1976).

However, other studies have shown that catecholamine levels were decreased in the penicillin-induced foci as compared to the contralateral mirror sites when spike activity was localized in the penicillin treated cortex; but no difference existed in both catecholamine levels between both foci when spike activity was fully propagated to the mirror focus (Kobayashi et al, 1976).

## E. SURGICAL ALTERATIONS IN BRAIN DOPAMINE

The destruction of central catecholaminergic neurons with 6-hydroxydopamine (6-OHDA) (Breese & Traylor, 1971), given intraventricularly, lowered the seizure threshold in rats (Bourn et al, 1972, Avalone & Samanin, 1977), and was also found to reduce significantly the anti-convulsant effect of carbamazepine (Quattrone & Samanin, 1977), a drug with a pharmacological profile similar to that of phenytoin (Theobald & Kunz, 1963, Stiener et al, 1970). However, bilateral lesions in the substantia nigra that produced a 58% reduction in whole brain dopamine, without affecting noradrenaline or 5-HT, failed to alter the threshold for minimal electroshock in rats (Adler et al, 1969). These findings suggested that striatal dopamine was not important in seizure susceptibility, but they were inconclusive because of the incomplete removal of striatal dopamine.

## F. EFFECT OF DOPAMINERGIC DRUGS ON SEIZURE THRESHOLD

### 1. Increased dopamine at receptors

#### (a) Intraventricular administration of dopamine

Dopamine, like noradrenaline, does not penetrate the blood-brain barrier. Administred intraventricularly to mice, it was shown to facilitate pentylenetetrazole seizures at low doses (0.5 - 1  $\mu$ g) and to antagonise such seizures at high doses (5 - 100  $\mu$ g). In rats, intraventricular dopamine was found to have no effect on electroshock seizure in doses ranging from 4 - 16  $\mu$ g, while a larger dose (32  $\mu$ g) had a seizure-facilitating effect (Browning & Maynert, 1971).

(b) Dopamine precursors

The administration of L-DOPA, the immediate precursor of dopamine, raised the central catecholamine levels, particularly dopamine. Boggan & Seiden (1971) reported that a 200 mg/kg dose of L-DOPA reduced the severity of seizure in DBA/2 mice. Killiam & Frey (1973) observed that the same dose elevated the threshold for maximal electroshock and pentylenetetrazol in both rats and mice. Several investigators observed that L-DOPA in combination with a monoamine oxidase inhibitor exerted anti-convulsant effects (Chen et al, 1968, McKenzie & Soroko, 1972). Some of the strongest evidence that dopamine played a role in seizure susceptibility was based on the ability of L-DOPA to reverse or at least antagonise, the seizure facilitating effect of reserpine (Azzaro et al, 1972, Boggan & Seiden, 1971). When used in combination with monoamine oxidase inhibitor, L-DOPA appeared capable of elevating a reserpine-depressed threshold to supranormal levels. These findings favoured dopamine over noradrenaline as mediator of the effect. However, uncertainty was interjected by observations that L-DOPA decarboxylation might occur extraneuronally (Bertler et al, 1966) and in serotonergic neurons (Chase et al, 1970, 1971). The possibility that dopamine acted as a 'false transmitter' at adrenergic and serotonergic sites could also be considered.

(c) Dopaminergic drugs

Increasing evidence suggested that apomorphine specifically activated dopamine receptors in the CNS (Ernst, 1966, Anden et al,

1967, Ungerstedt et al, 1969). McKenzie & Soroko (1972) found that apomorphine markedly elevated the threshold for maximal electroshock seizures in rats, but not in mice. Stull and his co-workers (1973) reported marked antagonism of RO-4-1284-induced facilitation of electroshock seizure by apomorphine and by intraventricularly administered dopamine. Since RO-4-1284 is a reserpine-like monoamine depleter, and the fact that the antagonistic effects of apomorphine and dopamine were completely blocked by pre-treatment with the dopamine-receptor blocking agent, pimozide, it was then suggested that dopamine did exert inhibitory effects in connection with electroshock seizure. These findings added apomorphine and dopamine to the reported substances that elevated seizure thresholds depressed by monoamine depletion and which included 5-HTP, monoamine oxidase inhibitors and noradrenaline. Thus, it would appear that almost any treatment that increased monoamine transmission would restore seizure threshold. Furthermore, the available information would suggest that restoration was more readily accomplished than elevation of a normal threshold to supranormal levels.

Apomorphine, ergocornine and piribedil were also shown to raise the seizure threshold in audiosensitive mice (Anlezark & Meldrum, 1975).

(d) Dopamine-releasing agents

It was shown that low concentrations of amphetamine released dopamine from the striatum almost as readily as noradrenaline from all its channels and that higher

concentrations were necessary to accomplish this effect at other dopaminergic nerve endings as those in the limbic region (Azzaro & Rutledge, 1973). It was shown that amphetamine protected audiosensitive mice from audio shock (Fuxe et al, 1974), and to reduce spike activity in the EEG of cobalt-implanted rats (Dow et al, 1974). However, other observations suggested that the released endogenous dopamine in brain might facilitate seizures (Spencer & Turner, 1969, Ellinwood et al, 1973).

(e) Effect of monoamine oxidase inhibitors and tricyclic anti-depressants.

Monoamine oxidase inhibitors, which elevated brain monoamine levels by inhibiting their catabolism, were found to raise the seizure threshold. The anti-convulsant action of monoamine oxidase inhibitors was thought to be mediated, at least in part, by an increase in the central levels of dopamine and noradrenaline (Fromm et al, 1973, Jarvik, 1970). Tricyclic anti-depressants acted by blocking the active reuptakes of monoamines at the nerve terminals, thus increasing the effective amine concentration at the synapse. It was reported that imipramine was effective in the treatment of some cases of petit mal and minor motor epilepsy (Fromm et al, 1973). However, many monoamine oxidase inhibitors and tricyclic compounds were too non-specific to permit differentiation of the effects of monoamine neurotransmitters.

2. Decreased dopamine at receptors

(a) Inhibitors of dopamine synthesis

Inhibition of tyrosine hydroxylase, the rate-limiting step in catecholamine synthesis, reduced the brain concentration of both transmitters. Alpha-methyltyrosine, the best known enzyme inhibitor, was observed consistently to increase the seizure susceptibility. Given alone, it lowered the threshold for tonic seizures in mice (Chen et al, 1968), antagonized the anti-convulsant action of acetazolamide (Rudzik & Mennear, 1966), and impaired the restoration of seizure thresholds in reserpine-treated mice (Wenger, Stitzel and Craig, 1973, Azzaro et al, 1972). These findings supported other evidence that reduced concentration of catecholamine increases seizure susceptibility but did not differentiate between the role of dopamine and noradrenaline.

(b) Dopamine blocking agents

Blockade of dopamine receptors with haloperidol or chlorpromazine facilitated seizures in low doses, but had an anti-convulsant effect at high doses (Chen et al, 1968). However, Killiam & Frey (1973), reported that haloperidol failed to influence chemically and electrically-induced seizures in mice.

(c) It has been shown that reserpine and tetrabenazine, which reduce the central levels of monoamine, lowered the seizure thresholds in a variety of models of epilepsy including pentylenetetrazol convulsions (Jones & Roberts, 1968, Pfeiffer & Galambos, 1965), electroshock (Azzaro et al, 1972), and



audio-iogenic seizures (Schlessinger, Boggan and Freedman, 1965, Lehman, 1967, Boggan & Seiden, 1971).

G. CATECHOLAMINES IN DIFFERENT MODELS AND ANIMAL SPECIES OF EPILEPSY.

All these data support the hypothesis that seizure threshold was linked to central catecholaminergic activity and this was further supported by microiontophoretic studies, some of which indicated that dopamine and noradrenaline acted as inhibitory transmitters in the cortex, (Frederickson et al, 1971, Jordan et al, 1972). However, some drugs like reserpine might well exert effects on more than one system. Moreover, it was established that functional links existed between noradrenaline and 5-HT systems in certain animals. It was found that sections of the dorsal noradrenaline bundle in the cat resulted in an increased cortical and raphe synthesis of 5-HT (Blondaux et al, 1975, Jouvet and Pujol, 1974). Furthermore, L-DOPA loading raised central levels of both dopamine and noradrenaline but have also been reported to reduce whole brain 5-HT in the rat, (Fuller & Penry, 1975). Moreover, drugs acting on noradrenaline and dopamine systems in brain might have an effect on intracerebral blood flow and hence might alter the regional metabolism not specifically related to catecholamine system (Edvinsson, 1975).

Some workers tried to separate the role of catecholaminergic and serotonergic systems in epilepsy. Kellog et al (1974), using the technique of catecholamine synthesis inhibition and precursor administration, found that seizure threshold in the audiosensitive mouse model could be raised by increasing and lowered by decreasing central catecholamine levels. Other workers have also reported that inhibiting



either tyrosine hydroxylase or tryptophan hydroxylase could delay the recovery of seizure threshold to control values after reserpine, whereas the simultaneous inhibition of both hydroxylases could prevent the recovery indefinitely. However, the threshold could be affected only by a combination of both synthesis inhibitors in normal mice (Azzaro et al, 1972, Killiam & Frey, 1970).

It was suggested that the neuronal substrate to epileptic behaviour was model-specific or species-dependent, and that the involvement of the monoamine system might differ widely from one model to another. In support of this view, it had been reported that audiogenic seizures in mice could be blocked by clonidine, an alpha-adrenergic antagonist, but not by apomorphine (Kellog et al, 1974), which stimulate the dopamine receptors. This is in contrast to the finding that clonidine aggravated while apomorphine reduced spike activity in cobalt-implanted rat (Ashcroft et al(1974). This might be related to the fact that morphine had a stimulant effect in mice, but a sedative effect in rats (Sellstrom et al, 1975). Other workers also suggested that various monoamine neurotransmitters played different roles in different models of epilepsy. In the mouse PTZ (pentylenetetrazol) model, it was shown that seizure threshold could be elevated by increasing and reduced by decreasing central 5-HT levels but was insensitive to changes in central catecholamine levels. However, the electroshock threshold in mice was found to be directly related to central noradrenaline and dopamine levels but unaffected by altering the serotonergic activity (Rudzik & Johnson, 1970). Similarly, apomorphine was shown to protect against maximal electroshock seizures (McKenzie & Soroko, 1972), while it potentiated PTZ seizure in mice (Soroko & McKenzie, 1970). Moreover, amphetamine was reported to raise the threshold for electroshock

in rabbits (De Schaepdryver et al, 1962), and mice (Frey, 1964), while lowering it in the rat (Jurnal & Regelby, 1968). These observations suggested that the circuitry of one type of convulsive model might be species-specific. The concept of model-specific pattern of neuronal involvement explains why many workers at present disagree with the early hypothesis of establishing a common biochemical aetiology, whether catecholaminergic (Swinyard et al, 1961, Chen et al, 1968), or serotonergic (Koe & Wiseman, 1966, Bonnycastle et al, 1957) to all types of epilepsy.

#### H. AIM OF THE WORK

Some workers tried to differentiate the role of both catecholamines, noradrenaline and dopamine, in epilepsy in order to provide a more specific answer. It was shown that phentolamine and sotalol, alpha and beta-adrenergic blockers respectively, had anti-convulsant effects in the mouse electroshock model (Navarro et al, 1974). In cobalt-induced epilepsy in the rat, Ashcroft et al (1974) demonstrated that apomorphine and amphetamine reduced or abolished spike activity in EEG of epileptic rats while spiroperidol and clonidine had the opposite effects. Moreover, the spike suppressant effect of dopamine receptor stimulants was blocked by spiroperidol, a specific dopamine-receptor blocker, which also initiated spike activity in control rats. However, these were few experiments utilizing few animals to demonstrate the drug effects. The present work was done to elaborate and extend the above work and to confirm possible aspects of the role of dopamine system in the cobalt model of focal epilepsy. Other dopamine agonists were used in the present study that had predominantly a direct stimulant action on dopamine receptors. The possible mechanism of action of

these drugs was investigated using some pharmacological and surgical manipulations of the dopamine system in the rat which might be helpful in uncovering the aetiological basis of this form of focal epilepsy.

## I. PHARMACOLOGICAL ASPECTS OF SOME DOPAMINE AGONISTS AND ANTAGONISTS

### 1. Apomorphine

It was well established that apomorphine administration to rats led to the production of stereotyped sniffing, licking and biting behaviour (Quinton & Halliwell, 1973, Randrup & Munkvad, 1968), probably due to stimulation of dopaminergic mechanisms in the striatum. Furthermore, apomorphine induced contralateral rotation in rats with unilateral degeneration of the nigro-striatal pathway induced by 6-hydroxydopamine (6-OHDA) (Ungerstedt, 1971, Dray & Oakley, 1976, Dray et al, 1975). Apomorphine-induced stereotypy and rotation began within 2 - 5 min and lasted about 60 min with peak rotation occurring about 30 min after the injection (Dray & Oakley, 1976). Both rotation and stereotypy could be blocked by the administration of pimozide (1 mg/kg.i.p.) or haloperidol (0.5 mg/kg.i.p.), either before or after the injection of apomorphine.

Apomorphine was also known to lower prolactin secretion in plasma of rats (Horowski et al, 1975), and to stimulate dopamine-sensitive adenylyclase (Kebabian & Greengard, 1972).

### 2. Bromocryptine

Bromocryptine or 2-bromo-alpha-ergocryptine is an ergot polypeptide derivative comprising a lysergic acid residue with a cyclic polypeptide moiety.

It was originally characterized as specific inhibitor of prolactin secretion in all the vertebrates tested including man (Fluckiger, 1972, Del Pozo et al, 1972). However, Hokfelt & Fuxe (1972) found that in the rat, bromocryptine also decreased dopamine turnover in the median eminence and suggested that it might interfere with the dopaminergic tuberoinfundibular neurons which control prolactin secretion.

It was then found that bromocryptine also decreased dopamine turnover in the neostriatum, an action attributable to direct stimulation of dopamine receptors at this site (Corrodi et al, 1973). This central dopaminergic stimulation was assumed to be responsible for the significant therapeutic action of bromocryptine in Parkinsonism (Calne et al, 1974a and 1974b) and in galactorrhea.

Bromocryptine was shown to induce a dose-dependent stereotyped behaviour in normal rats. Moreover, it induced a dose-dependent contralateral rotation in rats with unilateral lesion of the substantia nigra induced by 6-OHDA (Johnson et al, 1976). These effects of bromocryptine could be blocked by the prior administration of pimozide (1 mg/kg, i.p.) or haloperidol, and reduced by pre-treatment with reserpine or alpha-methyltyrosine (Johnson et al, 1976).

Moreover, bromocryptine stimulated spontaneous motor activity in normal mice (Johnson et al, 1976) in a dose-dependent manner. Bromocryptine produced its effects after a latent period of 50 to 70 min (Corrodi et al, 1973, Fuxe et al, 1974, Johnson et al, 1976, Dray & Oakley, 1976). Bromocryptine (5-20 mg/kg i.p.) induced—

rotation was fairly constant and persisted up to 240 min and declined over the next 5 - 7 hrs depending on the dose (Dray & Oakley, 1976, Johnson et al, 1976).

Dopamine-receptor stimulation by bromocryptine was also reflected by a reduction in cerebral dopamine turnover in the presence of alpha-methyltyrosine-induced synthesis blockade (Corrodi et al, 1973, Fuxe et al, 1974). The delay in the onset of action as well as the dependence on presynaptic events differentiated bromocryptine from the classical dopamine agonists. Bromocryptine also inhibited dopamine metabolism in man, and this was reflected by reduction of CSF levels of HVA in treated patients (Curzon, 1975).

Other neurotransmitter systems, such as 5-HT, were also affected by bromocryptine (Corrodi et al, 1975). 5-HT release was reduced, as reflected by 30-40% reduction of brain 5-HIAA in bromocryptine-treated rats (Snider et al, 1975), and reduction of 5-HIAA levels in CSF of treated patients (Curzon, 1975).

### 3. Lisuride hydrogen maleate

Lisuride hydrogen maleate is a derivative of isolysergic acid with strong peripheral anti-serotonin activity (Votava & Lamplova, 1961). This compound was synthesized by Zikan & Semonsky (1960) and was used mainly as a prophylactic agent in migraine.

Lisuride induced stereotyped behaviour in normal as well as in reserpinised mice. It antagonized the motor depression and hypothermia induced by reserpine. As with apomorphine, the

effects of lisuride hydrogen maleate were not impaired by pre-treatment with alpha-methyltyrosine. In untreated mice, the substance was very potent in producing a significant hypothermia with doses as low as 0.1 mg/kg i.p. The stereotyped behaviour and hypothermia could be prevented by pre-treatment with the dopaminergic antagonist haloperidol (Horowski & Wachtel, 1976). These actions indicate that lisuride hydrogen maleate was a potent dopaminergic agonist with a probably direct action on dopamine receptors. Furthermore, lisuride at doses as low as 0.1 mg/kg had a strong serum prolactin lowering effect in rats (Horowski et al, 1975, and in man (Horowski et al, 1977). In rats, Dlabac (1973) reported a marked increase in brain dopamine following an intravenous injection of 0.3 mg/kg lisuride hydrogen maleate and it was further shown that lisuride lowered the synthesis rate of dopamine, an effect which was also known for apomorphine (Kehr et al, 1975). In the dopamine-rich areas, the striatum and mesolimbic forebrain, lisuride (300-100 ug/kg) decreased the rate of L-DOPA formation in rats after inhibition of aromatic amino acid decarboxylase with NSD-1015, and this was counteracted by haloperidol (Kehr, 1977). In the predominantly noradrenaline-innervated neocortex, lisuride in doses of 0.3 and 1 mg/kg i.p. increased dopa accumulation. After inhibition of catecholamine synthesis with alpha-methyltyrosine, lisuride decelerated dopamine and accelerated noradrenaline disappearance. In addition, lisuride caused an increase in 5-HT and a decrease of 5-HIAA concentrations in the brain. Furthermore, lisuride reduced 3-methoxytyramine accumulation after monoamine oxidase inhibition with pargyline, suggesting inhibition of dopamine release. These data support the

views that lisuride stimulated post-synaptic dopamine and, to a lesser extent, 5-HT receptors and blocked noradrenaline receptors in the CNS (Kehr, 1977).

4. CF 25-397 [9,10-Didehydro-5-methyl-8-Beta (2-pyridylthio-methyl)ergoline].

This is a non-peptide ergot derivative which seemed to exert some dopamine-like action which differed from the effect of bromocryptine or L-DOPA and appeared to be more selective.

CF 25-397 induced a dose-dependent contralateral turning in rats with unilateral lesion in the substantia nigra, induced by 6-OHDA, from a dose of 1 mg/kg i.p. This effect was blocked by pre-treatment with pimozide (1 mg/kg i.p.) (Jaton et al, 1975). This indicated that the compound possessed central dopamine stimulating activity. However, even at high doses (30 mg/kg), stereotyped behavior was barely detectable. This suggests that its action on the nigro-striatal system was highly selective (Jaton et al, 1975). In mice, CF 25-397 inhibited reserpine-induced catalepsy. However, no motor stimulation was observed. On the contrary, CF 25-397 (2-5-10 mg/kg i.p.) slightly reduced the motor activity (Jaton et al, 1975). This drug also induced hypothermia in cold-acclimatized rats (Silbergeld et al, 1977). Like bromocryptine, the long latent period between the administration of CF 25-397 and the induction of its behavioural effects raised the possibility that the activity depends upon the conversion to active metabolites. Failure of CF 25-397 in clinical trials to



elicit any therapeutic effect in Parkinsonism might be explained by differences in drug metabolism between rats and man.

5. Pimozide

Blockade of dopaminergic receptor sites was widely implicated in the mechanism of action of various anti-psychotic agents. Neuroleptics, such as the diphenylbutylpiperidine derivative, pimozide, appeared to be a selective blocker of the dopamine receptors (Janssen, 1967, Anden, Butcher and others, 1970). Studies have suggested that blockade by anti-psychotics of dopamine receptors in the striatum and the mesolimbic system were closely related with the anti-psychotic action of these drugs (Anden, 1972, Anden & Stock, 1973).

6. 6-hydroxydopamine (6-OHDA)

The stereotaxic injection of 6-OHDA in discrete brain areas has been widely used to produce selective and specific degeneration of catecholamine neurons. This method proved a valuable tool in morphological (Maler et al, 1973, Ungerstedt, 1971) and functional (Agid et al, 1973., Lidbrink & Fuxe, 1973., Ungerstedt, 1971., Arbuthnott, 1970., and Ungerstedt, 1970) studies.



## 1. ANIMALS

Male Wistar rats (200-250 g in weight) were prepared with cobalt implants and recording electrodes as already described in Methods of Section 1.

## 2. THE STEREOTAXIC FIXATION OF STAINLESS STEEL SCREW CANNULAE FOR INTRASTRIATAL INJECTION OF DRUGS

In some animals, prior to the implantation of cobalt-gelatin sticks, two specially constructed stainless steel cannulae (especially made in the Department) were fixed, one to each side of the skull, so as to enable injection of drugs into the striatum.

Using the stereotaxic frame, a point (7.7 mm anterior to the interaural line, and 2.7 mm lateral to the midline), was located on each side of the skull (Konig & Klippel, 1963). The skull was trephined, and the hole centered at this point, using No. 6 burr. One hollowed screw cannula was fitted to the skull on each side at this point, and fixed with acrylic cement (Simplex). Injections were made 3.5 mm deep to the cortical surface (Konig & Klippel, 1963), using 18 gauge needle. The solution for injection was freshly prepared and drawn into a special 2 ml glass syringe (Aglar) which was then fitted into a micro-meter which will control the volume of the injected solution. The needle length was such that it could traverse the hollowed screw cannula and project 4.5 mm below its tip so as to be stereotaxically placed in the striatum (i.e. A 7.7, 2.7 L, and V 3.5 mm) (Konig & Klippel, 1963).

### 3. TECHNIQUE FOR INTRA-STRIATAL INJECTION

Injectons of 6-OHDA were made into each side of the striatum in the anaesthetized rat. One  $\mu$ l of solution was injected slowly. An interval of 30 sec was allowed before injecting the next 1  $\mu$ l. This process was continued until the required volume of solution was given.

For experiments with dopamine agonists, injections were 10 - 16 days after operation when epileptic foci following cobalt implantation were well established. At that time, the rat was handled in such a way that its head was straight and parallel to the plane of the table. Injections were made into the striatum as described above, care being taken to prevent movement of the rat head during the injection procedure. At the end of the recording procedure, and before each injection, care was taken to avoid blocking of the hollowed screws by blood mixed with CSF which might have leaked after the injection, clotted and solidified.

### 4. ELECTROCORTICOGRAPHIC (ECoG) RECORDING

#### A. Instrumentation

ECoG recordings were made from the unrestrained conscious rats. Twisted connectors, attached to braided wire, were fitted into the hollow bore of the recording stainless steel screw electrodes. The brain activity was amplified and recorded on a Grass polygraph, (Model 7). During each session, ECoG recording was done from two rats, one of which was drug-treated while the other served as a control.

Four channels were utilized and each amplified and recorded the potential difference between two of the electrode links, as follows:-

Channel 1:

The right frontal (active) and right posterior (parietal) electrode.

This would record the potential difference due to epileptic activity in the right frontal cortex, i.e. the primary focus of the first rat.

Channel 2:

The left frontal electrode (active) and the left posterior (parietal) electrode.

This would record the epileptic activity in the secondary focus in the left frontal cortex of the first rat.

Channel 3:

The right frontal electrode (active) and the right posterior electrode, i.e. primary focus of the second rat.

Channel 4:

The left frontal electrode (active) and the left posterior electrode, i.e. secondary focus of the second rat.

Channels 1 and 2 were amplified through direct current (D.C.) preamplifiers which utilized a transistor chopper with a time constant of 0.8 sec. Direct current (D.C.) amplification was used in order to enable the recording of any changes in the characterization of the low frequency component of the ECoG which might have changed in epileptic rats. All channels had high frequency filters

set on the driver amplifiers at 35 Hz, and they all utilized a 50 Hz mains filter to block any interference from other equipment. The final recording sensitivity was 100  $\mu$ V/cm from all channels, and the paper chart speed was set at 10 mm/sec. Before each recording, the polygraph was calibrated from a low frequency oscillator (SLEE, model 81), producing a balanced sine wave signal so that all pens were deflecting equally at a frequency of 8 Hz. Input voltage of 100  $\mu$ V was fed from the oscillator into each channel at the level of the grid connections, and the preamplifier sensitivity adjusted to give 1 cm per deflection.

Calibration was essential so that direct comparisons of the ECoG amplitudes could be made between all channels.

#### B. ECoG Recording Technique

The rat, with previously inserted electrodes, was placed in a perspex box with the recording leads attached. It was allowed to settle for 10 min in the new environment, a perspex cover was placed on top of the case to prevent the animal from escaping. During this settling period, the calibration signal of 100  $\mu$ V sine wave, at a frequency of 8 Hz, was recorded for 1 min. The paper speed was 10 mm/sec. After this settling period, the leads from electrodes attached to the animal were connected to the grid, and ECoG recording was done from the conscious unrestrained rats.

These recordings could be made within 24 hrs after the implantation of cobalt. In order to follow the development of epileptic activity in cortical foci in the brain, recordings were done for 10 min either daily or on alternate days in the first week,

and then on alternate days during the second week after cobalt implantation, depending on the nature of the experiments.

However, to detect the effect of drugs on the epileptic activity of cortical foci, long recordings were required. Initially, a 30 min ECoG recording was done followed by the administration of the drug. Recording was then continued for 2 hrs or longer after drug administration, depending on the nature of the drug under study. Such recordings were made 8 - 16 days after cobalt implantation when the motor manifestation and activity of cortical epileptic foci were prominent.

Recording sessions were standardized as far as possible particularly with regard to the time of the day and the sequence of recording the individual animal in any group, since there have been reports of diurnal fluctuation of seizure threshold on various models of epilepsy (Schreiber & Schlessinger, 1971, Wooley & Timiras, 1962). In general, ECoG recordings were carried out in the morning. In drug experiments, the drug injection was normally given between 9.30 - 10.00 a.m.

During the 10 min recording, the behaviour of the animal and epileptic motor manifestation were noted. During drug experiments, the stereotyped behaviour and motor epileptic manifestations were noted every 15 min following the drug injection. However, detailed behavioural rating was not done. Attempts were made during the recording to prevent the connecting leads from becoming entangled as the animal moved about the recording box, by having the 4 leads

braided into a harness. However, if the rat continued to circle in one direction, the leads tended to become twisted to such an extent that recording had to be stopped, the leads removed and straightened before continuing the recording. For routine recording, this precaution was very satisfactory, but it was noted more when animals were treated with dopamine agonists such as apomorphine. However, this did not interfere to any extent with the continuation of the recordings.

Sometimes, it was also necessary to prevent the rat from pulling the leads down with its forefoot, and chewing through them or actually eating them. This can be prevented by gently poking the animal with a wooden or rubber stick, or tapping the top of the cage. Theoretically, this has the disadvantage of changing the ECoG characteristics by arousing the animal, but in the present work, this was done infrequently so as to avoid disturbing the animal to the extent of interfering with the ECoG characteristics.

## 5. AUTOMATIC ANALYSIS OF ELECTROCORTICOGRAM

### A. Instrumentation

The bipolar ECoG recording from each cerebral hemisphere of the rat were amplified through the Grass polygraph (Model 7), using 35 Hz filters, 0.03 second time constant, and also recorded on  $\frac{1}{4}$ " magnetic tape, using a Tandberg I.R. (Series 100) tape recorder, at a speed of  $1\frac{1}{8}$ " / sec. At this recording speed, the Tanberg bandpass is from 0-62 Hz. These tapes were used subsequently for computer analysis and spike counting, according to the method of Hill & Townsend (1973), which utilizes the criterion of sharpness,

i.e. peak angles. The signals were then analyzed by replaying the tape at the recording speed through an analogue low pass filter with a band width of 25 Hz. The band limited signal was sampled at a rate of 200 samples/sec/channel, and digitised to a 7-bit (1%) amplitude accuracy. Gain and offset controls were used to ensure full utilization of the dynamic range of the analogue digital computer.

The computer used was an Elliot 905 connected on-line to a 1-16 channel analogue to digital (A-D) and digital to analogue (D-A) convertor, via a special 8-bit parallel interface (NPL modified). An Elliot series 20 visual display enabled information to be displayed and a TSP-212 point plotter was used to produce permanent graphical records. Input-output was on 8 hole punched paper tape, and all analyses were carried out in real time.

The recognition of spikes by the computer was done by separating the recorded phenomena into a set whose members were 'spikes' and a complementary set of 'not spikes'. The rest of the ECoG like waves, muscle activity and electrical artefacts did not undergo the same mapping as spikes.

For long recordings, a programme has been designed to monitor recordings each 5 min, i.e. total spike count per 5 min. For 10 min recordings, the programme averages the spike counts during that period.

#### B. Epileptic spike recognition

The main EEG or ECoG features of epilepsy is the spike. The

International Federation has already defined a 'spike' as a 'wave distinguishable from background EEG activity and having a duration of one twelfth second or less', and a 'sharp wave, as a wave distinguishable from background EEG activity, with a duration of more than one twelfth second and less than one fifth second'. These criteria fitted the results from the rat model very well as the frequency of epileptiform episodes occurring at any given time was much more predictable in the rat than in recording from epileptic patients (in whom epileptiform spikes are sparse and unpredictable, and can be easily inhibited). It has been found that even the process of connecting a patient to the EEG machine has some inhibitory effect on epileptic phenomena (Hill & Townsend, 1973). The same inhibitory phenomenon does occur in rats when one connects them first to the recording leads before putting them in the recording box. However, this lasts about 10 min and, since routinely 10 min period was allowed for the animals to settle, this did not pose a problem.

There have been attempts to utilize computers for the recognition of epileptic spikes in the EEG and ECoG (Saltzberg et al, 1971, Carrie et al, 1972). These methods rely on empirically-adjusted threshold values and therefore cannot be used easily as routine procedure. The development of an automatic spike recognition was itself complicated at the outset by the fact that there is often disagreement between observers on what is a real epileptiform activity and what is not when EEG records are inspected visually. Mosley et al (1972) laid down six guidelines, of which three were helpful in solving this difficulty. These included:-



1. Every spike-looking wave is to be regarded as artefact, unless there are good reasons for suspecting otherwise.
2. Epileptically significant spikes and sharp waves are almost always surface negative in polarity initially, or at least, the sharpest or highest voltage component of the wave is usually surface negative.
3. Sharp or spiky events which can be logically explained by simple alterations in voltage of the background rhythm or by superimposition of several components in the background activity of the record should be ignored.

Taking these guidelines into account, Hill & Townsend (1973) described a computer programme which allowed the automatic detection and estimation of the number of spikes in the rat ECoG and human EEG. This was done by measuring the peak angles of each wave in the ECoG sample recordings. Spike recognition by measuring the peak angle required that a criterion should be established such that waves with peak angles smaller than the criterion angle were classified as spikes.

In addition to peak angles, other reference features in classifying spikes are the rise time and fall time (Kooi, 1966). However, these are difficult to estimate because the precise points of inflection (at the start and finish) are not easy to define, since they depend on the form of the underlying activity and its phase relationship to spikes.

### C. Measurement of peak angles by the computer

The rate of change of potential during the rising and falling phase of the spike is approximately linear and a straight line can be fitted by the least squares method to give an accurate estimate of the gradients. With a sampling rate of 200/sec, it was found that four points exclude very few spikes and make the method highly artefact-resistant.

The angle between straight lines which are fitted by the computer to four points on either side of the peak is the peak angle (Fig. 13A). This angle was computed and its size used in analysis of ECoG recordings. The effect of low pass filtering is to round the peak and slightly slow the rise and fall times. The ease with which two waves can be distinguished by measurement of their peak angles is dependent upon the gain. This effect is already known in recognising human spikes, whereas if the gain is increased or the recording paper speed is decreased, the record appears more spiky, while conversely at low gains or fast paper speeds, the spikes may be difficult to recognise. For this reason, the amplitude value used in calculating the peak angle must be corrected by reference to a suitable calibration signal.

### D. Distribution of peak angles

If the statistical distribution of peak angles is examined by performing a histogram, it is found in the case of records with clearly visible spikes that the distribution is bimodal (Fig. 13B). This suggests that it consists of two populations:-

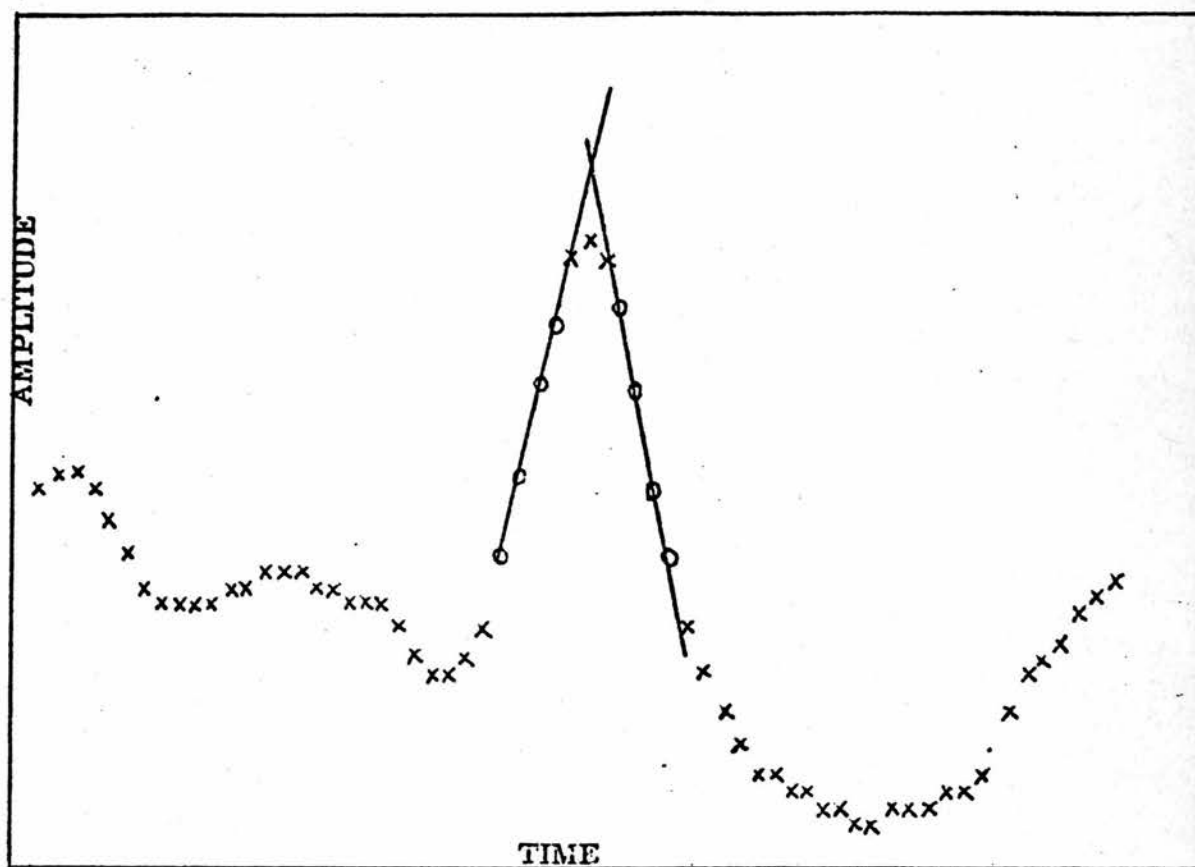


Fig. 13 A Calculation of Peak Angle of a Wave

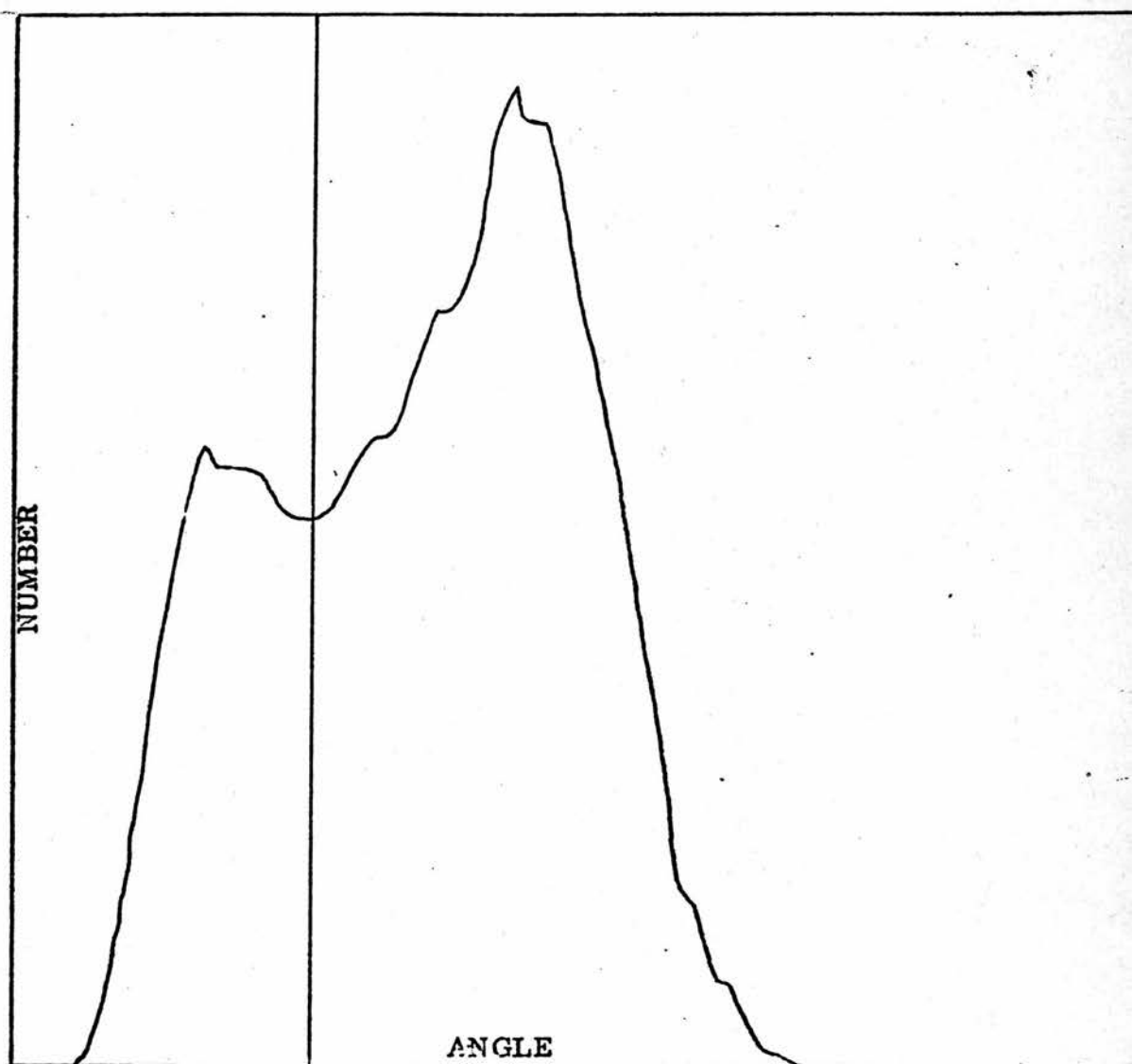


Fig. 13 B Histogram of Peak Angles

- (a) SPIKES - these constitute the small groups on the left with acute angles.
- (b) WAVES - these are the larger groups with less acute angles.

The trough between the two peaks of distribution is then used to determine the criterion peak angle. Thus, spikes form a distinct population which can be detected and measured with suitable accuracy to enable monitoring the ECoG correlate of epileptic focus during its development and under the influence of drugs.

#### 6. DRUG SOLUTIONS, DOSES AND MODE OF ADMINISTRATION

The following drugs were used during experimentation:-

(a) Apomorphine hydrochloride (Evans)

This was available in ampoules containing 3 mg/ml. It was used in doses of 0.5, 1 and 2 mg/kg, given by intraperitoneal (i.p.) injection.

(b) Bromocryptine mesylate (Sandoz)

This drug was freshly dissolved in a few drops of glacial acetic acid and 0.5% ethanol and the required volume made with 5% glucose solution so as to give a concentration of 20 mg/ml. The drug was given i.p. Control rats received an equivalent volume of the vehicle i.p.

Care was taken that the volume of solution injected was less than that required for ethanol to produce any sedative effect or affecting catecholamine metabolism (Carlsson & Lindquist, 1973, Carlsson, Engel, Strombom, Svenson & Waldeck, 1974).

Bromocryptine was used in doses of 10 and 20 mg/kg i.p.

(c) Lisuride hydrogen maleate (Schering)

This drug was freshly dissolved in normal saline to make a final concentration of 1 mg/ml. Lisuride was injected (i.p.) in doses of 0.1, 0.25, 0.5 and 1 mg/kg.

(d) CF 25-397 (9,10-didehydro-6-methyl-8B-(2-pyridylthiomethyl) ergoline) (Sandoz)

CF 25-397 powder was freshly dissolved in a few drops of glacial acetic acid and the required volume made with 5% glucose water to make a concentration of 40 mg/ml. This drug was given (i.p.) in doses of 20 and 40 mg/kg.

(e) Pimozide (Janssen Pharmaceuticals Co)

This drug was freshly dissolved in a few drops of glacial acetic acid, neutralized with one drop of 0.5% ethanol and the final volume made with 5% glucose water to a concentration of 2 mg/ml. This drug was injected (i.p.) in a dose of 1 mg/kg.

(f) 6-hydroxydopamine (6-OHDA) (Sandoz)

This powder was freshly dissolved in normal saline to make a concentration of 10 mg/ml, and containing ascorbic acid (0.5 mg/ml) to avoid its oxidation. This drug was administered in a dose of 20 µg into each striatum.

(g) Dopamine (Sandoz)

This powder was freshly dissolved in normal saline to make a 12.5 mg/ml solution, which was used for intrastriatal injection in a dose of 25  $\mu$ g (2  $\mu$ l) in one or both striata.

(h) Apomorphine HCl powder (Sandoz)

This powder was freshly dissolved in normal saline to make a solution of 30 mg/ml containing 0.5 mg/ml ascorbic acid to avoid its oxidation. This drug was injected in a dose of 60  $\mu$ g (2  $\mu$ l) into each striatum.

7. EXPERIMENTAL DESIGN AND GROUPING OF RATS

A. EFFECT OF DOPAMINE AGONISTS ON FOCAL EPILEPTIC ACTIVITY  
OF CORTICAL FOCI INDUCED BY COBALT IMPLANT

1. Effect of apomorphine

Animals were divided into 3 groups, each group of 4 cobalt-implanted rats for testing the effect of 0.5, 1 and 2 mg/kg of apomorphine respectively.

ECoG recording was done for a control period of 30 min from each pair of rats. One rat, then, received the assigned dose of apomorphine (i.p.) while the control rat was injected (i.p.) with an equal volume of normal saline. Recording was continued for 2 hrs following the administration of the drug.

2. Effect of bromocryptine

Eight rats were used, being divided into 2 groups of four animals each. One dose of bromocryptine mesylate;

either 10 or 20 mg/kg (i.p.) was assigned to each group to test its effect on epileptic activity when cortical foci were well established.

ECoG recording was done for a control period of 30 min from each pair of rats. Then one rat received bromocryptine (i.p.) while the other animal received an equivalent volume of the vehicle similarly. Recording was then continued every second half hour for the following 6 hours after the injection, observing the epileptic and behavioural changes every 15 min. This is due to the reported latency (50-60 min) for onset and the prolonged action of bromocryptine in comparison to other dopamine agonists such as apomorphine. ECoG recordings of 30 min duration were also done after 24 hrs to test whether the effect of the drug had lapsed.

3. Effect of lisuride-hydrogen maleate on cortical epileptic foci

Fourteen rats were used. These were divided into 4 groups, two of which were of 3 rats each, while each of the other two groups contained 4 cobalt-implanted rats. Each group was used for testing the effect of one dose of lisuride either 0.1, 0.25, 0.5 and 1 mg/kg respectively. The procedure was the same as that described for apomorphine, except that the animals received a dose of lisuride (i.p.) after 30 min control period of ECoG recording.

4. Effect of CF 25-397 on focal epileptic activity

Seven Wistar rats were used, being divided into two groups of 3 and 4 animals for testing the effect of 20 and 40 mg/kg of



the drug, respectively. The procedure is the same as that described for bromocryptine, except that the animals received CF 25-397 in the assigned dose after an initial 30 min period of ECoG recording.

B. EFFECT OF DAILY ADMINISTRATION OF BROMOCRYPTINE (20 mg/kg INTRAPERITONEALLY) ON THE DEVELOPMENT OF SPIKE ACTIVITY IN EPILEPTIC CORTICAL FOCI IN COBALT RATS

Starting on the first day after cobalt implantation, bromocryptine was given daily (20 mg/kg) i.p. to one group of 5 rats, while the control group received an equivalent volume of the vehicle daily (i.p.).

ECoG recording for 10 min was done prior to the injection daily for the first week and then on alternate days until the 12th day.

C. EXPERIMENTS WITH DOPAMINE RECEPTOR BLOCKER, PIMOZIDE, ON FOCAL CORTICAL EPILEPTIC ACTIVITY INDUCED BY COBALT IMPLANTATION

1. Effect of pimozide alone

Three pairs of rats were used for this experiment. ECoG recording was done for a control period of 30 min. Then one rat received pimozide (1 mg/kg) i.p. while the control rat received an equivalent volume of the vehicle (i.p.). Recording and behavioural observation was continued for 2 hrs following the injection.

2. The effects of pimozide on the actions of dopamine agonists

This was assessed in a series of experiments in which pimozide (1 mg/kg) or an equivalent volume of vehicle (for control rats) was given (i.p.) 45 min before the intraperitoneal administration of apomorphine (2 mg/kg) or lisuride (0.1 mg/kg) and 20 min before bromocryptine (20 mg/kg) or CF 25-397 (40 mg/kg) i.p. ECoG recording and assessment of the central effects were continued as described above with each respective agonist.

#### D. EXPERIMENTS ON THE STRIATUM IN COBALT IMPLANTED RATS

##### 1. Effect of bilateral intrastriatal injection of dopamine on epileptic activity in cobalt-implanted rats

When cortical epileptic foci were well established after 8-16 days of cobalt implantation, ECoG recording was done for a control period of 20 min from each pair of rats. One animal received, then 2  $\mu$ l (25  $\mu$ g) of freshly prepared dopamine solution (12.5 mg/ml) into each striatum, while the control rat received 2  $\mu$ l saline bilaterally into the striatum. ECoG recording and behavioural observation were then continued for one hour after the injection, noting changes in epileptic manifestations as well.

##### 2. Effect of bilateral intrastriatal injection of apomorphine on epileptic activity

The design of the experiment is similar to that described for dopamine except that the treated rat received 60  $\mu$ g (2  $\mu$ l) apomorphine HCl solution, freshly prepared, while the control rat received an equivalent volume of saline into each striatum,

as described in the Methods.

3. Effect of unilateral injection of dopamine into the striatum on focal epileptic activity

The experimental design is the same again (1) but the treated rat received 2  $\mu$ l (25  $\mu$ g) of dopamine into the right striatum while the control animal received 2  $\mu$ l saline into the same striatum, as described in the methods.

4. Effect of bilateral destruction of catecholamine terminals in the striatum with 6-hydroxydopamine (6-OHDA) on the development of focal epileptic cortical foci in cobalt-implanted rats.

A. Procedure

Twelve rats received cobalt-implant into the right frontal cortex. At the time of the operation 2  $\mu$ l (20  $\mu$ g) solution of 6-OHDA (10 mg/ml) was injected slowly into each striatum of 6 rats, while the remaining 6 rats received 2  $\mu$ l normal saline into each striatum.

The rats were observed daily for behavioural changes and epileptic manifestation. Many of the rats required supplementary feeding due to hypokinesia and difficulty in eating and drinking in the post-operative period.

Daily ECoG recording was started on the first day after operation, recording was done for 10 min from one drug-treated and one control animal at a time. Tissues from lesioned and control rats were assayed together. ECoG recording was

continued daily for the first 8 days and then on alternate days for the following week.

B. Dissection of striatum

Two weeks after the operation, the rats were killed by decapitation, their brains quickly removed on a mixture of solid CO<sub>2</sub> and crushed ice. The striatum on each side was quickly dissected out, as described in the methods. Tissues were quickly stored in liquid nitrogen for subsequent biochemical estimation of dopamine and noradrenaline in striatum. Biochemical analysis was done within 4 weeks of freezing in liquid nitrogen.

C. Biochemical estimation of dopamine and noradrenaline

Noradrenaline and dopamine were measured in the rat striatum using the enzymatic isotopic method (Palkowitz et al, 1974), which is a modification of the radiometric catechol-O-methyl transferase assay for catecholamines described by Engelman & Portnoy (1970). The reported sensitivity (twice the blank) of the assay was approximately 15 picograms (pg) for noradrenaline and 45 pg for dopamine (Palkovitz et al, 1974).

Tissues were homogenized in 300 µl of 0.1 N perchloric (HClO<sub>4</sub>) acid. After centrifugation, the supernatant was taken into 15 ml glass centrifuge tubes. Blanks contained 300 µl of 0.1 N perchloric acid. Standards were prepared by adding 50 µl of 0.1 N HCL O<sub>4</sub> containing 25 ng dopamine or noradrenaline (free base) to 300 µl brain extract.

The reaction was initiated by the addition of 100  $\mu$ l of a mixture containing 500  $\mu$ g of dithiothreitol, 0.5  $\mu$ M magnesium chloride, 140  $\mu$ M of tris-HCL (pH 9.6) 25  $\mu$ l catechol-O-methyl transferase, and 2.5  $\mu$ l of  $^3$ H-S-adenosyl-1-methionine methyl (SAM). (Specific activity 500 mCi/mMol, Radiochemical Centre, Amersham). Incubation was continued for 50 min at 37°C and the reaction was stopped by the addition of 500  $\mu$ l of 0.5 M borate buffer, pH 10. Fifty microliters (  $\mu$ l) of a non-radioactive carrier solution, containing 7  $\mu$ g methoxytyramine, 3  $\mu$ g normetanephrine, 3  $\mu$ g metanephrine and 1 mg EDTA, was added to each sample. The O-methylated products were then extracted into a 9 ml of water-saturated ethyl acetate:methanol (10:1 V/V) by shaking for 30 sec. The phases were then separated by a centrifugation at low speed and 8.5 ml of the organic phase was transferred to another tube containing 0.5 ml of 0.5 M borate buffer, pH 10, to remove the phenolic acids. After shaking for 30 sec and centrifuging, the organic phase was transferred to a third tube containing 0.5 ml of 0.1 N HCL into which the O-methylated products were extracted into the aqueous phase by shaking for 30 sec. The tubes were then centrifuged for 5 min, and the organic phase was aspirated and discarded. The acid phase was then washed by the addition of 8 ml of water-saturated ethyl acetate and followed by mixing for 30 sec. After centrifugation, the ethyl acetate was aspirated and discarded. The tubes were then transferred to ice bath and 0.5 ml of 0.5 M sodium phosphate buffer, pH 7.5, was added to each tube.

To separate dopamine from noradrenaline, the side-chain of the latter was cleaved at the  $\beta$ -hydroxy-position with 50  $\mu$ l of freshly

prepared 3% sodium metaperiodate solution. The reaction was allowed to proceed for 3 min and was then stopped by the addition of 50  $\mu$ l of 10% glycerol. The [ $^3\text{H}$ ]methyl vanillin was then extracted into 10 ml of toluene by shaking for 30 sec, followed by centrifugation to separate the phases. Nine ml of the toluene phase was then transferred to another tube containing 1 ml of 0.1 N NaOH for the noradrenaline assay, while the aqueous phase was left for the dopamine assay.

The [ $^3\text{H}$ ]methyl vanillin in the 9 ml toluene phase was extracted in 0.1 N NaOH by shaking for 30 sec and the organic phase was removed after centrifugation. The aqueous phase was then acidified with 0.1 ml of glacial acetic acid and the vanillin was again extracted into 10 ml of toluene. Nine ml of the organic phase was then transferred to a counting vial containing 0.4 ml liquiflour in scintillator counter. The counting efficiency of tritium was estimated to be 34%.

The aqueous phase of the metaperiodate reaction was used for dopamine determination. Five ml of toluene were added to each tube, mixed for 30 sec, centrifuged and the organic phase aspirated off. This was followed by the addition of 0.5 ml of 1 M borate buffer pH 11 and 5 ml of toluene:isoamyl alcohol (3:2 V/V) to each tube, shaking for 30 sec and centrifuging at low speed to separate the phases. Five ml of the organic phase were then transferred to scintillation vial to which 10 ml of scintillant (NE 260) was added and counted for [ $^3\text{H}$ ] in scintillation counter.

The important characteristics of this assay are:-

1. The  $\beta$ -hydroxylated catecholamine (e.g. epinephrine) will also be measured in the noradrenaline assay. However, since epinephrine is present in trace amounts in the rodent brain (Iversen, 1967), this was not a limitation for the assay. Catechol precursors of noradrenaline, particularly dopamine, did not cross-react to any marked degree. Furthermore, it has been shown that both assay procedures exhibited linearity with amounts of catecholamines ranging between 10 pg and at least 10 ng (Coyle & Henry, 1973).
2. The toluene wash reduced the contamination of dopamine by noradrenaline to about 5-7% (i.e. 5-7% of the count number obtained for a given amount of noradrenaline in the noradrenaline assay will be obtained in the dopamine assay). However, the cross contamination of the noradrenaline assay by dopamine is about 1%. So, ideally, neither assay should be done in the absence of the other in order to correct for cross contamination of one amine by the other unless one can predict beforehand that cross contamination will be insignificant because of preponderance of one amine to the other. In the striatum where the assay was done in the present piece of work, dopamine is present in high concentrations in comparison to the trivial amounts of noradrenaline; so, the cross contamination between the amines is negligible.
3. The dithiothreitol should be stored dessicated at 4°C. The reagent should be weighed and added to the reaction mixture at the time the latter is prepared. A stock solution of dithiothreitol should not be used.

4. It is better to leave behind a small amount of the organic phase when it is aspirated and discarded, rather than to lose any of the products present in the aqueous phase.

#### Calculations

$$\begin{array}{l} \text{ng/g wet tissue weight} \\ \text{(dopamine or noradrenaline)} \end{array} = \frac{25 \times \text{dpm of sample}}{\text{dpm of standard}} \times \frac{1000}{\text{tissue weight (mg)}}$$

Sample cpm (counter per min) = Counted cpm minus cpm of reagent blank.

For dopamine, reagent blank ranged between 882 and 974 counts/min and standard cpm was calculated to be 34334.

For noradrenaline reagent blank ranged between 155 and 216 counts/min and the standard cpm was calculated to be 15398 counts/min.

The estimated counting efficiency for tritium was 34%.



## RESULTS

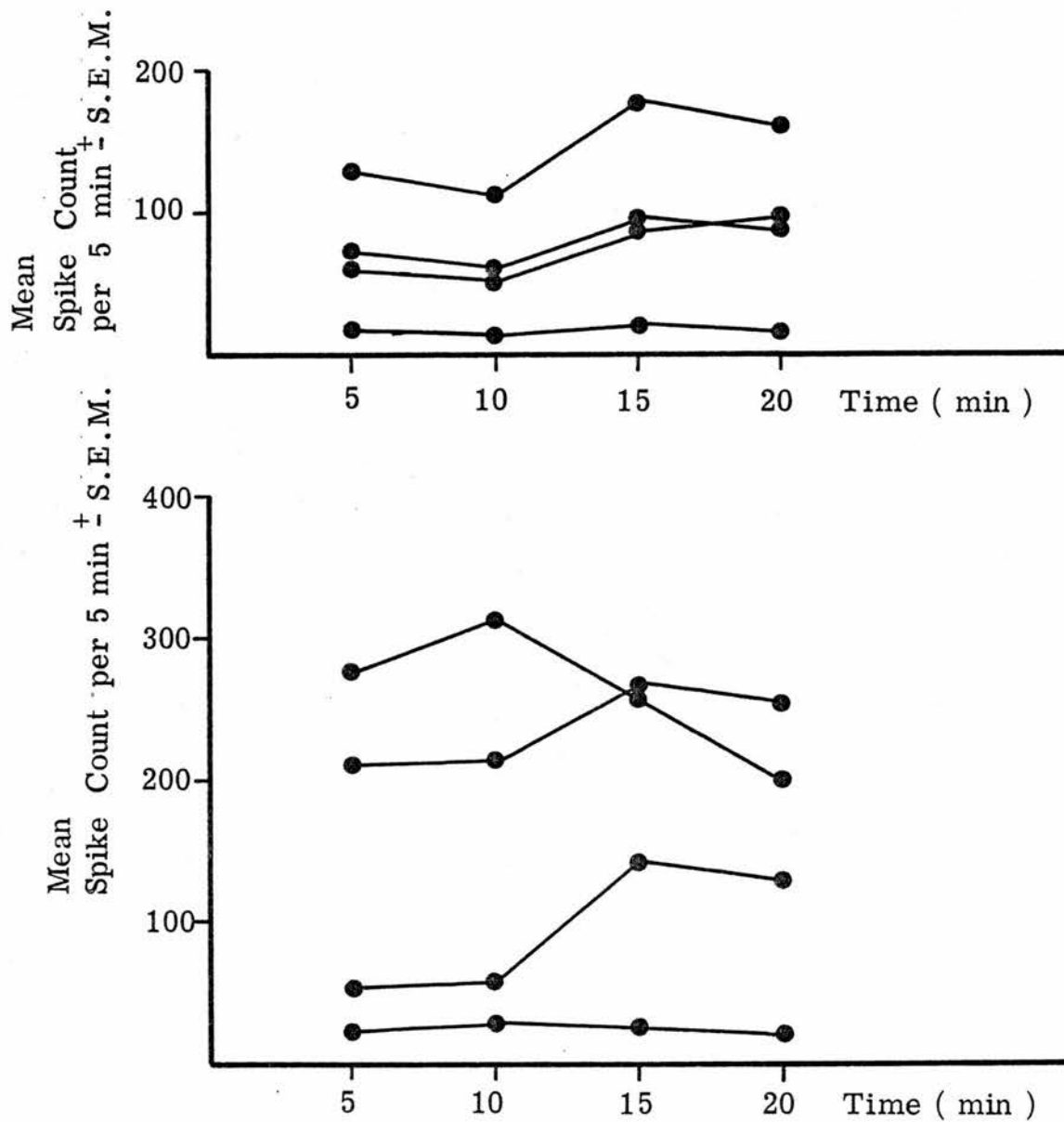
In all experiments involving periods of long ECoG recording, it has been noticed that the spike frequency of cortical foci vary considerably between different animals. This is illustrated in Fig.14 which shows the spike frequency per 5 min of different rats in two different groups of experiments during an initial control period of 20 min ECoG recording.

This animal variation precluded using the computed spike counts per 5 min as such for quantitative comparison of the effect of various drugs on ECoG recorded activity of different rats. This is because what might have been a spike suppressing effect, induced by a drug in one animal, would be a normal control epileptic activity in another. Accordingly, it was important to eliminate this animal variation by normalising the initial control period of 30 min for all rats. This was done by expressing the average spike count per 5 min during that period as a ratio of one for each rat, and then calculating the subsequent drug effect on each 5 min spike activity of either the primary or secondary cortical foci as a fraction of one. This will clearly show the effect of each drug on spike activity in each animal. Besides, it will allow evaluation of the severity of spikes suppression by various doses of anti-convulsants. The spike ratios, thus estimated every 5 min, were compared between drug-treated or saline (or vehicle)-treated controls, using the non-parametric Mann-Whitney-U-test.

However, with experiments involving daily 10 min recordings from cobalt implanted rats, comparison was done using the actual spike counts per min and the Kruskal-Wallis non-parametric, one-way analysis of

Fig 14

Spike Activity of Cortical Foci in Two Different Groups of Rats During a Control Period of 20 Minutes.



variance.

#### A. EFFECT OF DOPAMINE AGONISTS

##### 1. Effect of apomorphine on epileptic activity

(See Figs. 15a, b, c, d). Apomorphine produced a significant suppression in epileptic activity of both primary and secondary cortical foci induced by cobalt (Mann-Whitney U-test) which was evident within a few minutes of its intraperitoneal administration. This effect lasted for 30, 40 and 55 minutes with doses of 0.5, 1 and 2 mg/kg respectively (See Fig. 15), and was found to be significantly dose-dependent (Kruskal-Wallis non-parametric one-way analysis of variance).

For primary focus      $H = 7.84$     $P = 0.01-0.02$

For secondary focus    $H = 9.1$ ,    $P = 0.01-0.02$ .

This spike suppressing effect was also associated with suppression of motor epileptic manifestation (whiskers and forelimb twitches) which paralleled the duration of drug action.

Apomorphine also induced behavioural changes of central dopaminergic stimulation in the form of increased motor activity, arousal, and stereotyped gnawing, sniffing, licking, and sometimes biting which also corresponded to the duration of drug action.

The effect of apomorphine was followed by a period of reduced motor activity and rebound increase in spike activity of variable duration. However, this rebound reached significance level with a dose of 2 mg/kg intraperitoneally ( $P = 0.05$ ) in comparison to saline-treated animals, but generally the animals returned to normal behaviour after 1.5-2 hrs of drug administration. The saline-injected controls generally showed a tendency towards an

Legend to Fig. 15

EFFECT OF APOMORPHINE ON EPILEPTIC ACTIVITY IN COBALT-IMPLANTED RATS.

n = number of rats

- Each bar represents the mean spike ratio per 5 min  $\pm$  standard error (S.E.M.).

1. ECoG recording was done for a control period of 30 min. The average computed spike count per 5 min during that period from either the primary or secondary focus, was represented as a ratio of 1.

2. Apomorphine was injected intraperitoneally at 30 min.

Fig. 15A : 0.5 mg/kg apomorphine was injected  
B : 1 mg/kg apomorphine was injected  
C : 2 mg/kg apomorphine was injected  
D : Saline was injected into control rats  
(Pooled effect).

3. ECoG recording was continued for 2 hrs after the injection.

During this period of each animal, each spike ratio per 5 min represents the computed spike count per 5 min from either the primary or secondary focus expressed as a fraction of its average count per 5 min during the initial control period of 30 min.

4. Comparison of effect between drug-treated and saline-injected animals was done with the Mann-Whitney-U-test.

\* P < 0.002  
+ P = 0.002 - 0.02  
 $\pm$  P = 0.02  
+- P = 0.02 - 0.05  
++ P = 0.05

5. Kruskal-Wallis one-way analysis of variance showed the effect of apomorphine to be dose-dependent (P = 0.01 - 0.02).

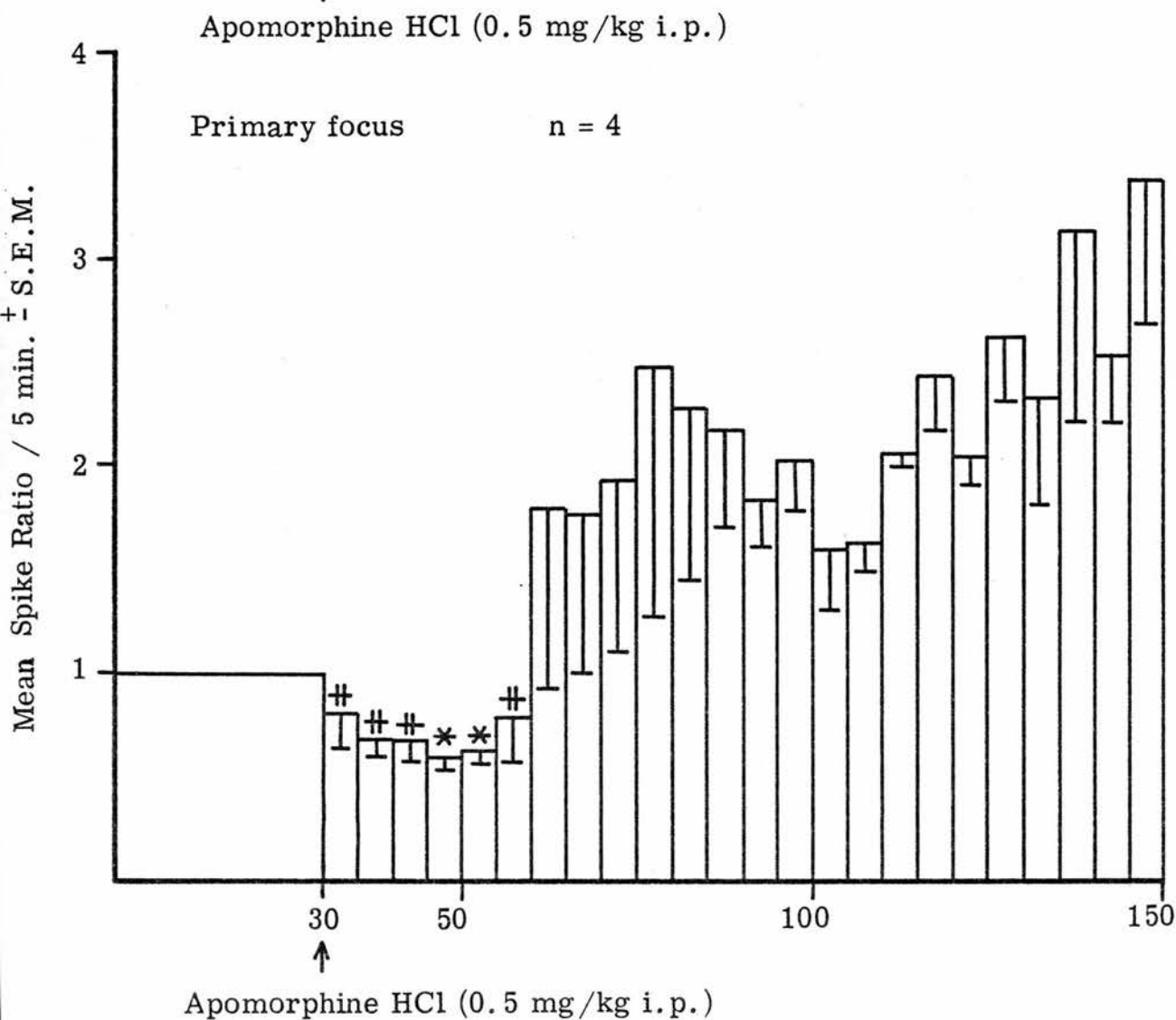
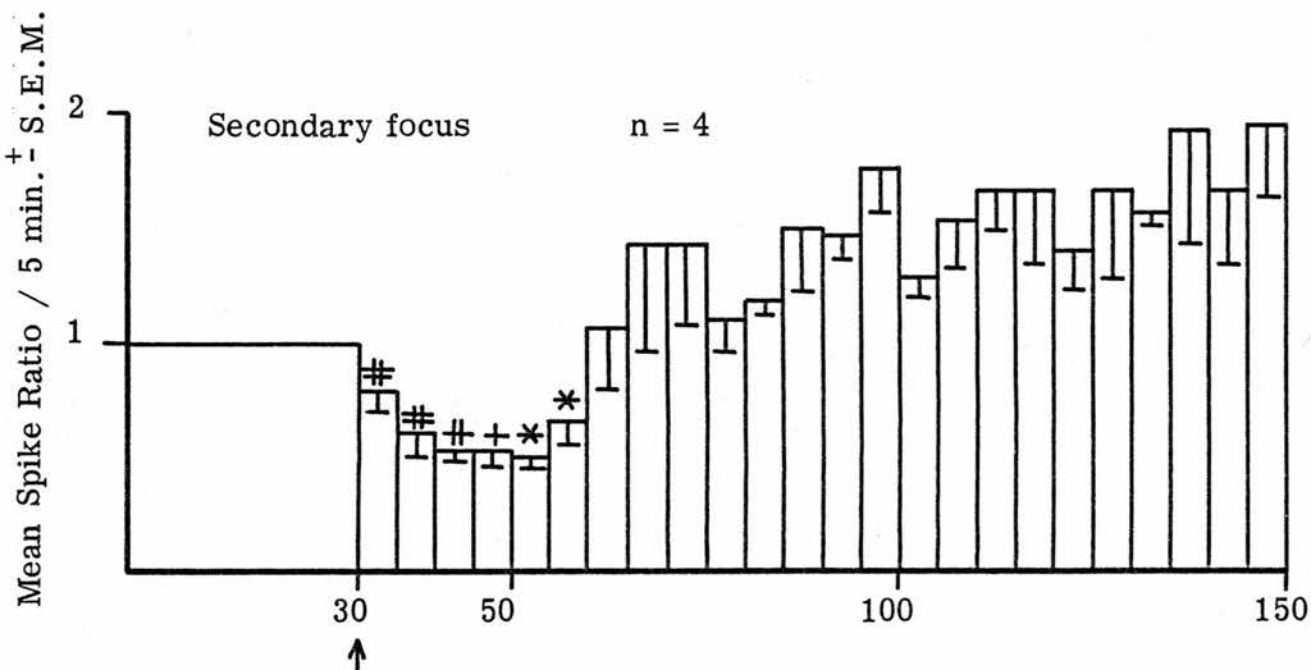


Fig 15B

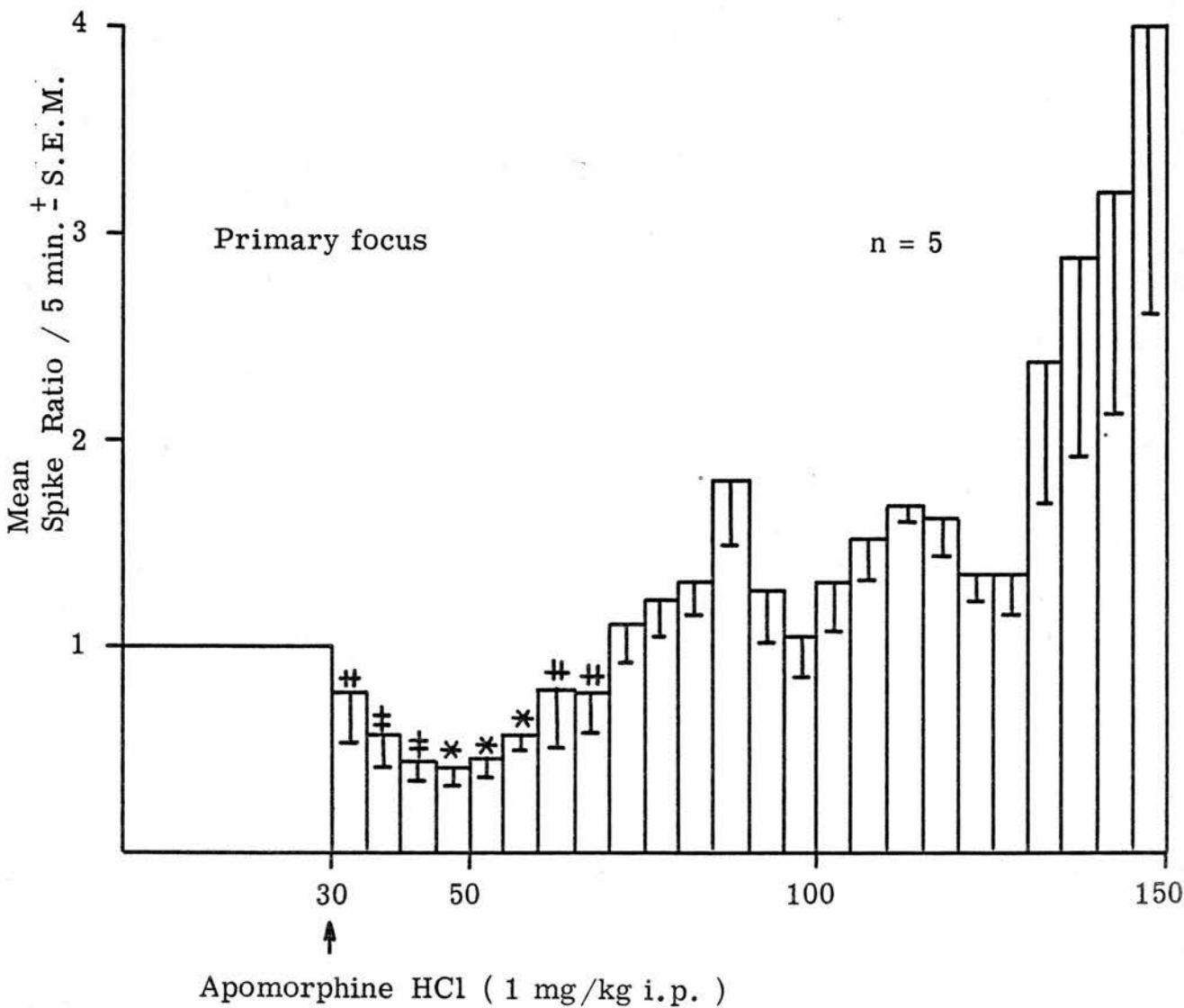
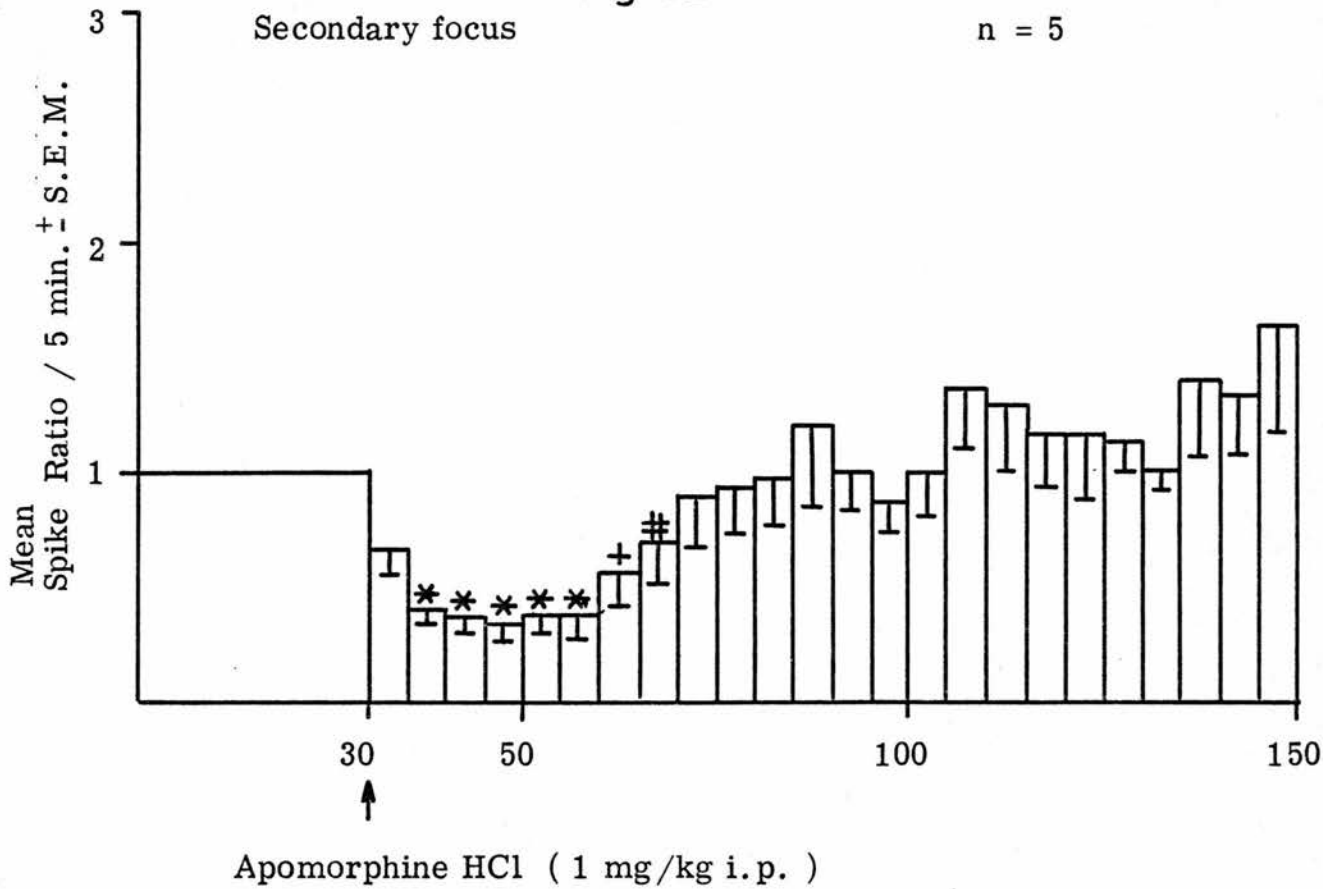


Fig 15C

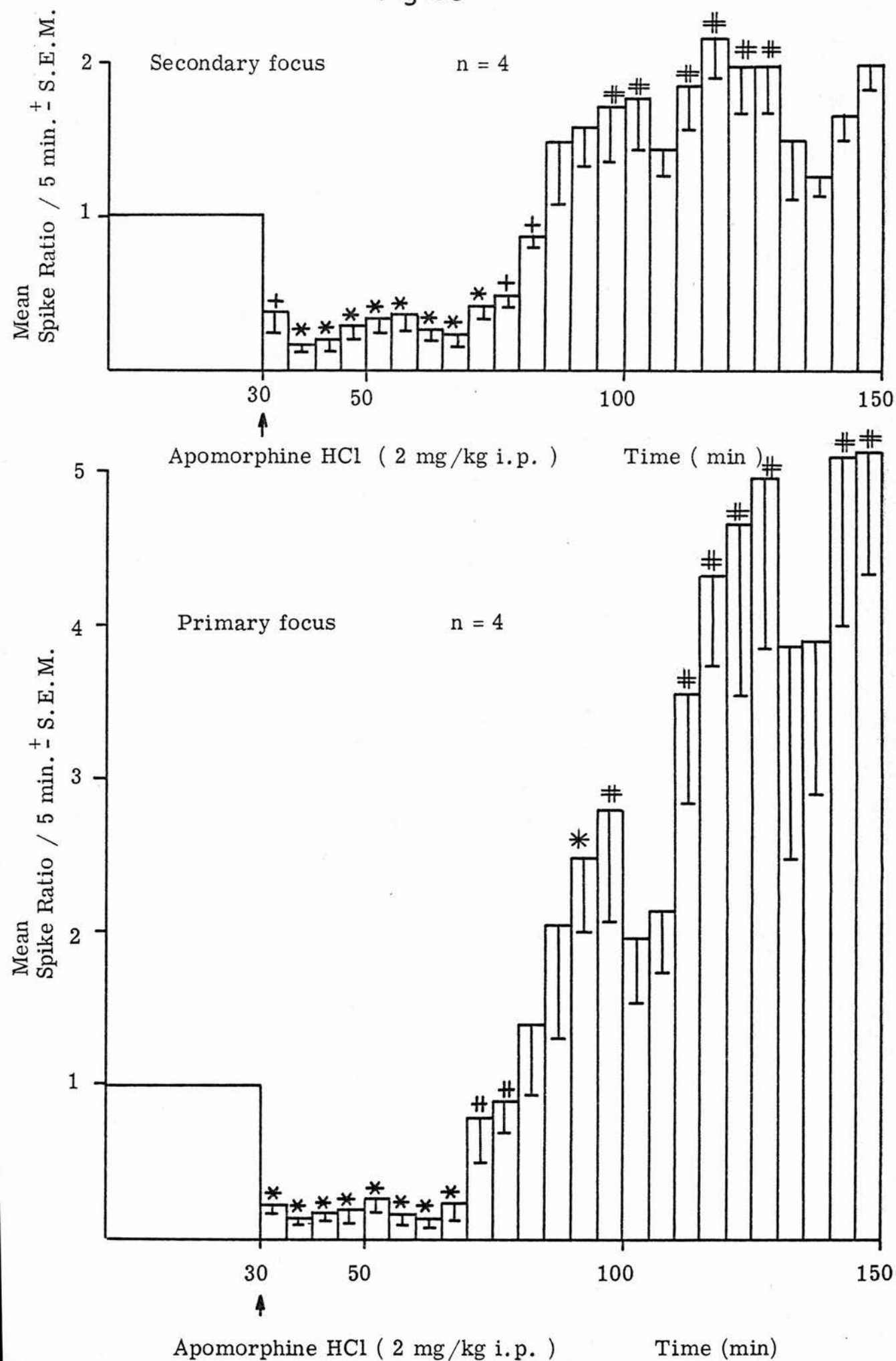
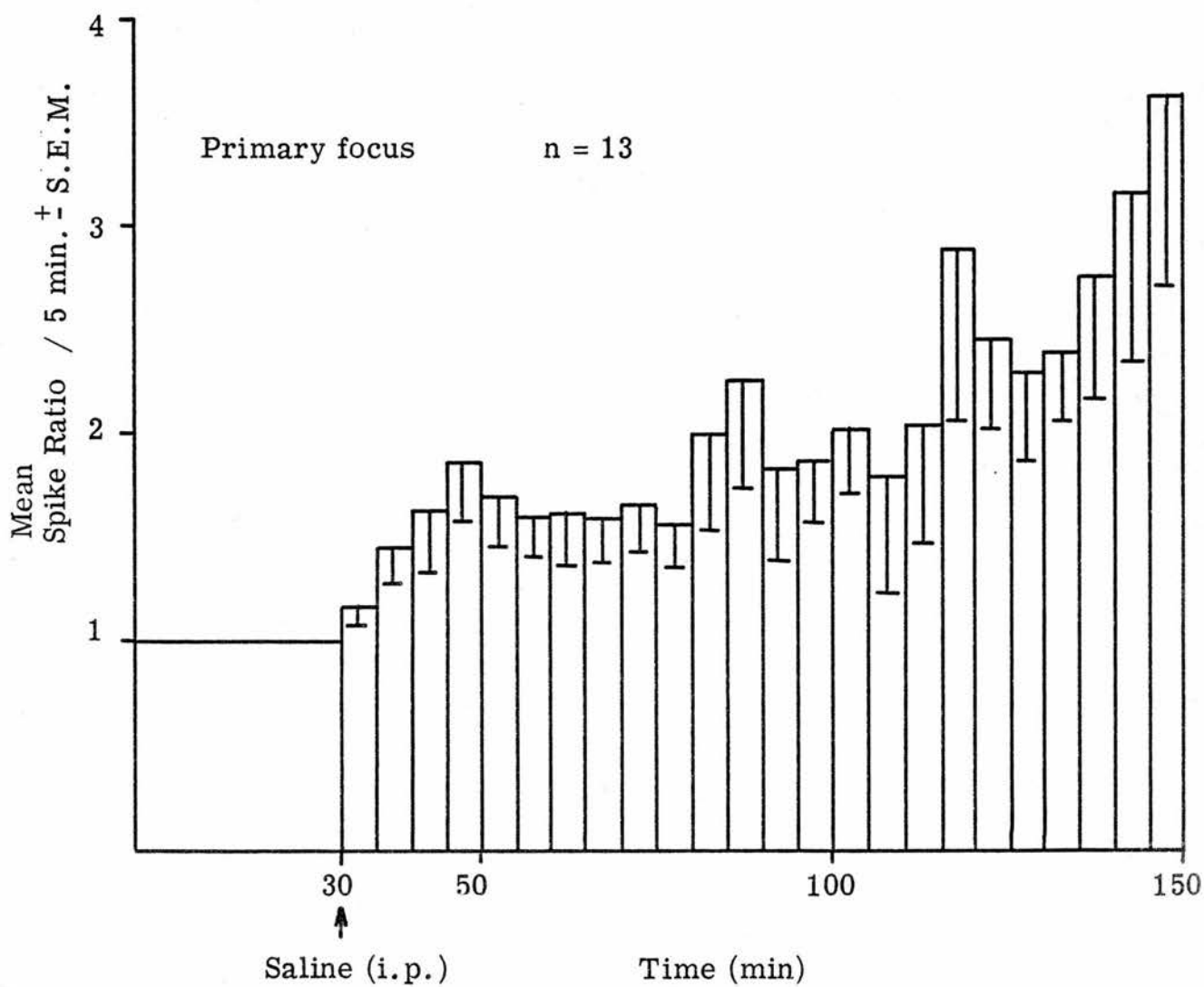
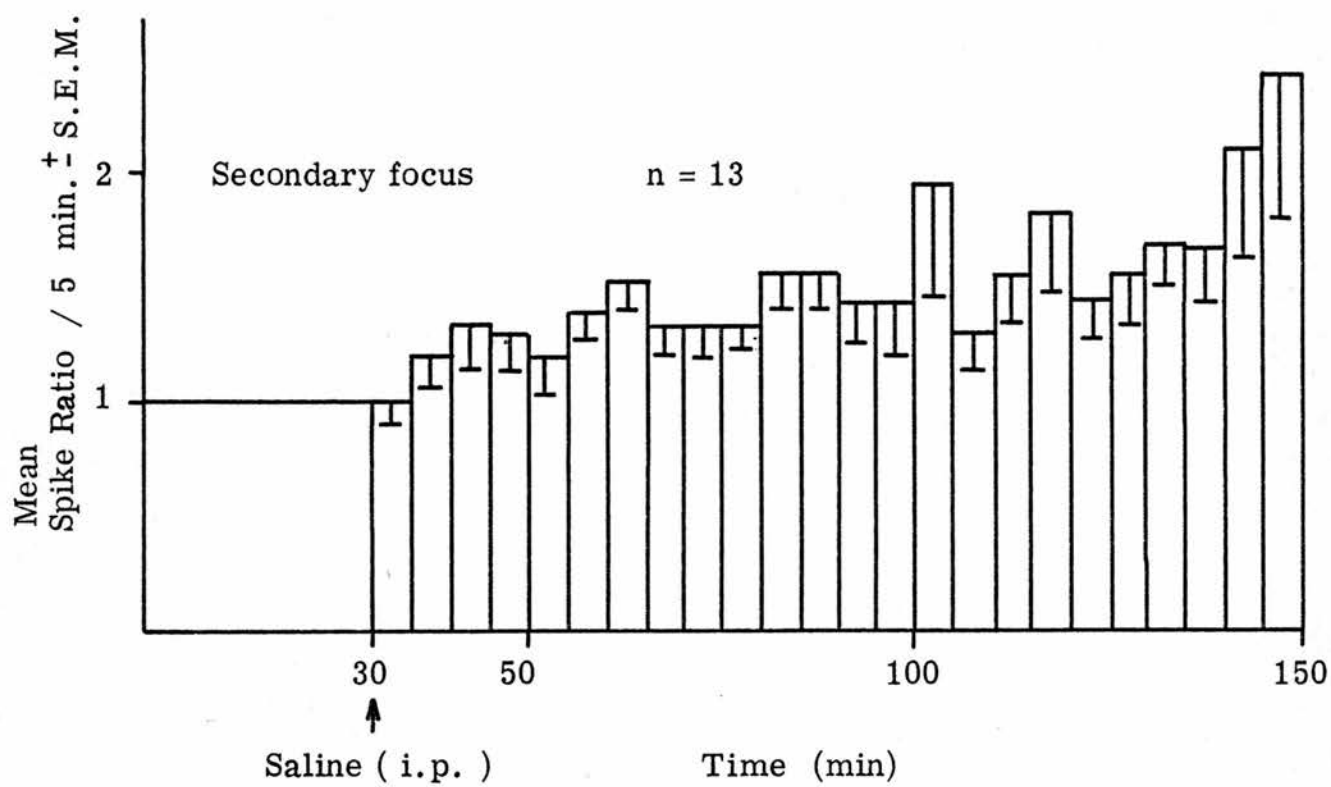


Fig 15D





increase in spike activity of cortical foci, but showed no change in behaviour.

## 2. Effect of bromocryptine on epileptic activity

(See Figs. 16a, b and c). Bromocryptine induced a significant (Mann-Whitney-U-test) reduction in epileptic activity which was evident within 3 hrs of intraperitoneal administration of 10 mg/kg, and within 1 hr of giving 20 mg/kg i.p. The effect of bromocryptine was marked 4 hrs after the injection (See Figs, above), and was found to be dose-dependent (Kruskal-Wallis one-way analysis of variance).

For primary focus       $H = 13.7$ ,       $P < 0.001$

For secondary focus     $H = 18$ ,       $P < 0.001$ .

The effect of bromocryptine was accompanied by a reduction in forelimb twitches and whiskers in the treated rats, as well as increased locomotion, arousal, and stereotyped gnawing, sniffing and licking.

The effect of bromocryptine on epileptic activity and behaviour was still evident 6 hrs after its administration. However, 24 hrs after the injection, the drug effect had disappeared.

The vehicle-injected controls showed no alteration in epileptic activity or behaviour.

## 3. Effect of lisuride on epileptic activity

(See Figs. 17a, b, c, d and e). Lisuride hydrogen maleate produced a significant reduction in epileptic activity of primary

Legend to Fig. 16

EFFECT OF BROMOCRYPTINE ON EPILEPTIC ACTIVITY IN COBALT-IMPLANTED RATS

n = number of animals

- Each bar represents the mean spike ratio per 5 min  $\pm$  standard error (S.E.M.).

1. ECoG recording was done for an initial period of 30 min. The average computed spike count per 5 min during that period from either the primary or secondary focus was represented as a ratio of 1.

2. Bromocryptine was injected intraperitoneally at 30 min.

Fig. 16A : 10 mg/kg bromocryptine was injected  
16B : 20 mg/kg bromocryptine was injected  
16C : Equivalent volume vehicle was injected into controls (pooled results).

3. ECoG recording was continued every second half hour for the following 6 hrs after the injection. Recording was also done for 30 min after 24 hrs.

During the post-injection period of each animal, each spike ratio per 5 min represents the computed spike count per 5 min from either focus, expressed as a fraction of its average count per 5 min during initial control period of 30 min.

4. Comparison between drug-treated and control animals was done with the Mann-Whitney-U-test.

\* P = 0.004

+ P = 0.008

≠ P = 0.016

≠ P = 0.028

≠ P = 0.048

5. Kruskal-Wallis analysis of variance showed the effect of bromocryptine to be significantly dose-dependent ( $P < 0.001$ ).

Fig 16 A

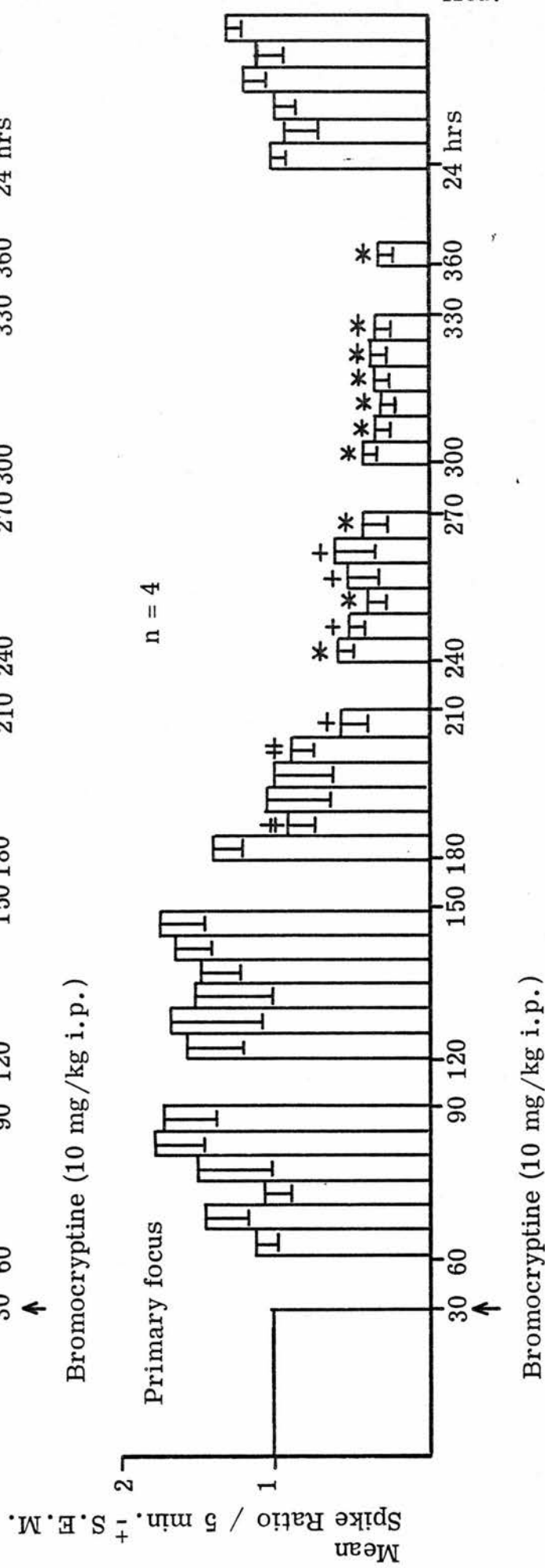
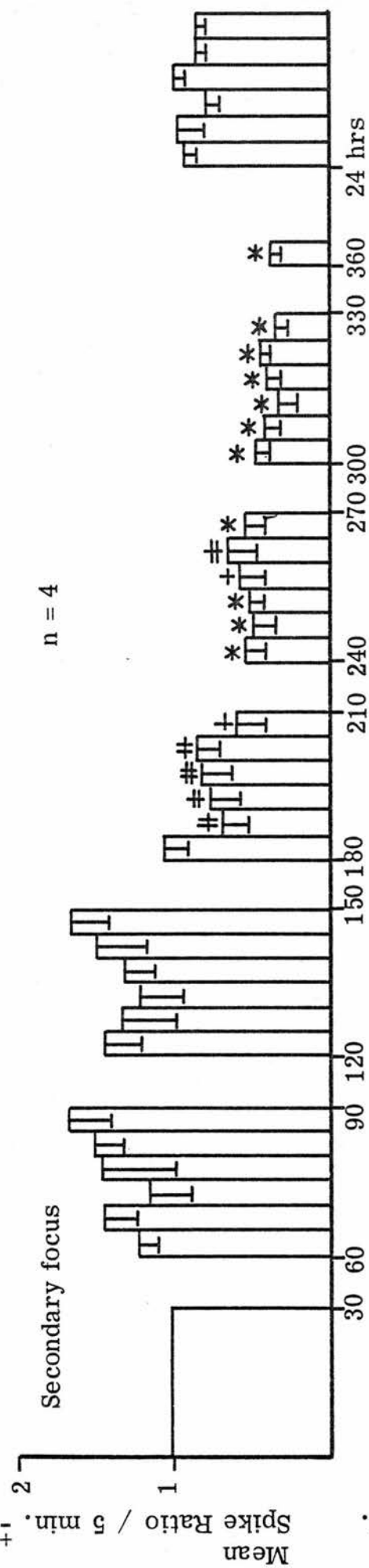


Fig 16 B

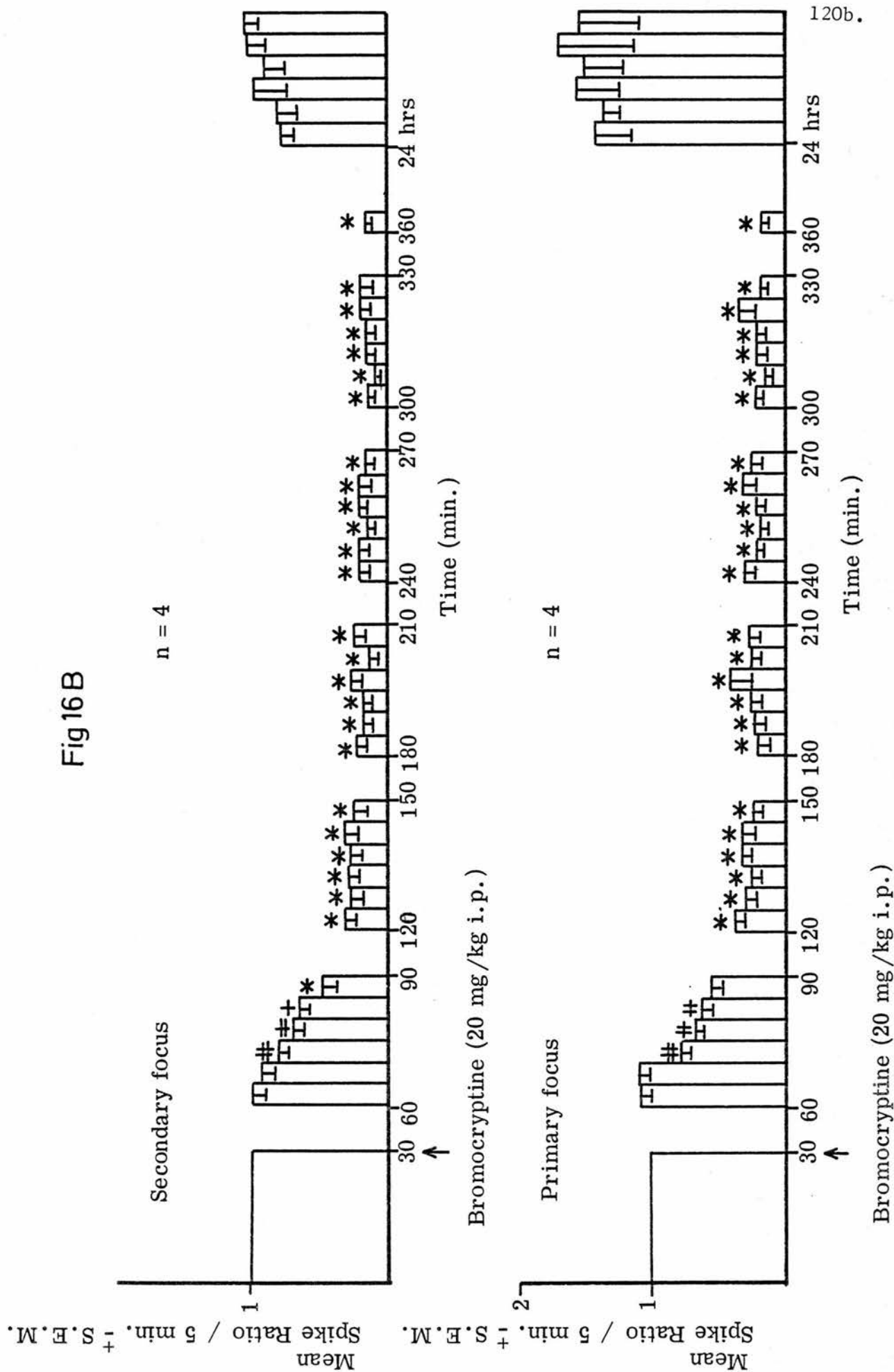


Fig 16C<sub>1</sub>

Pooled Controls for Effect of Bromocryptine

n = 8

Primary focus

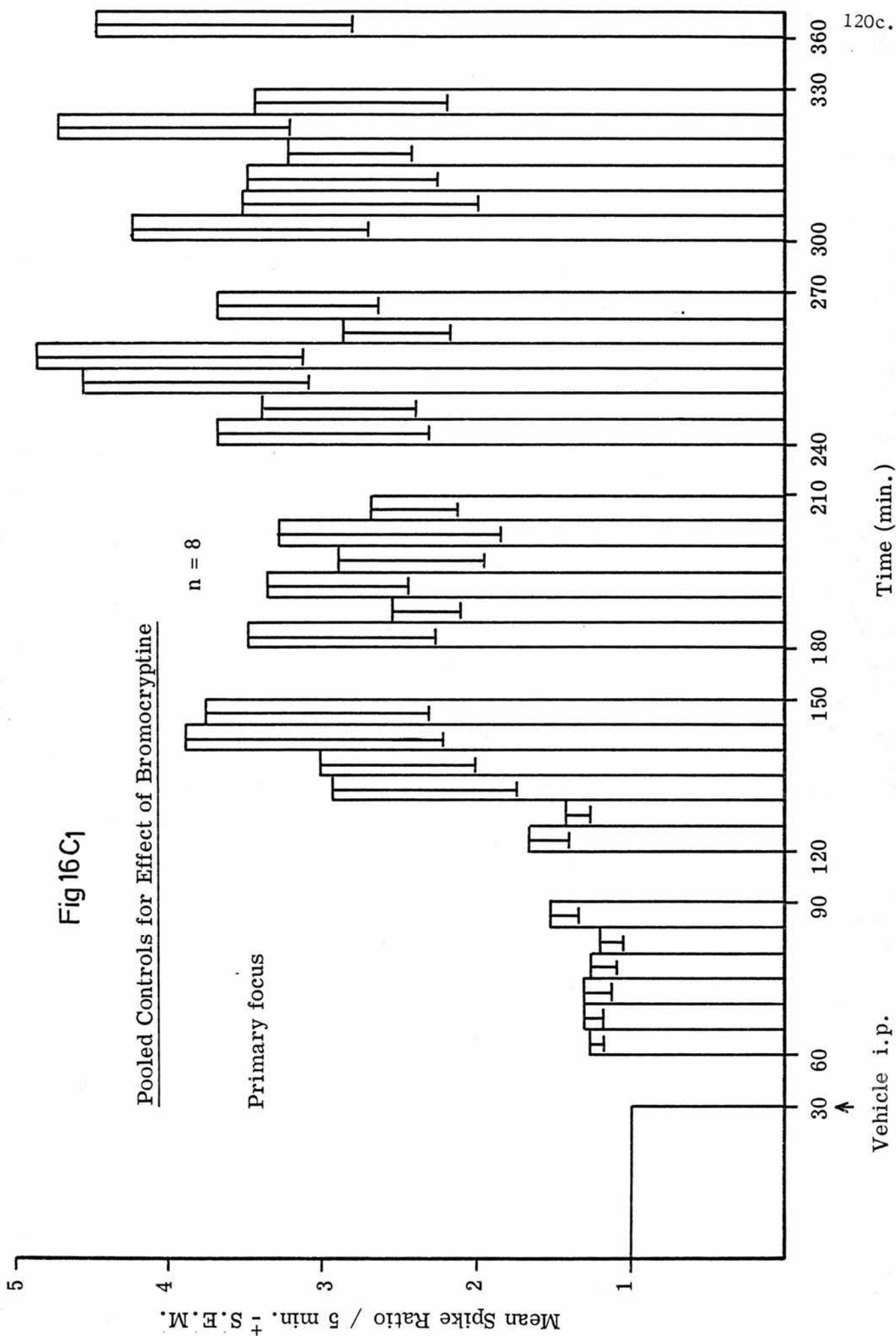
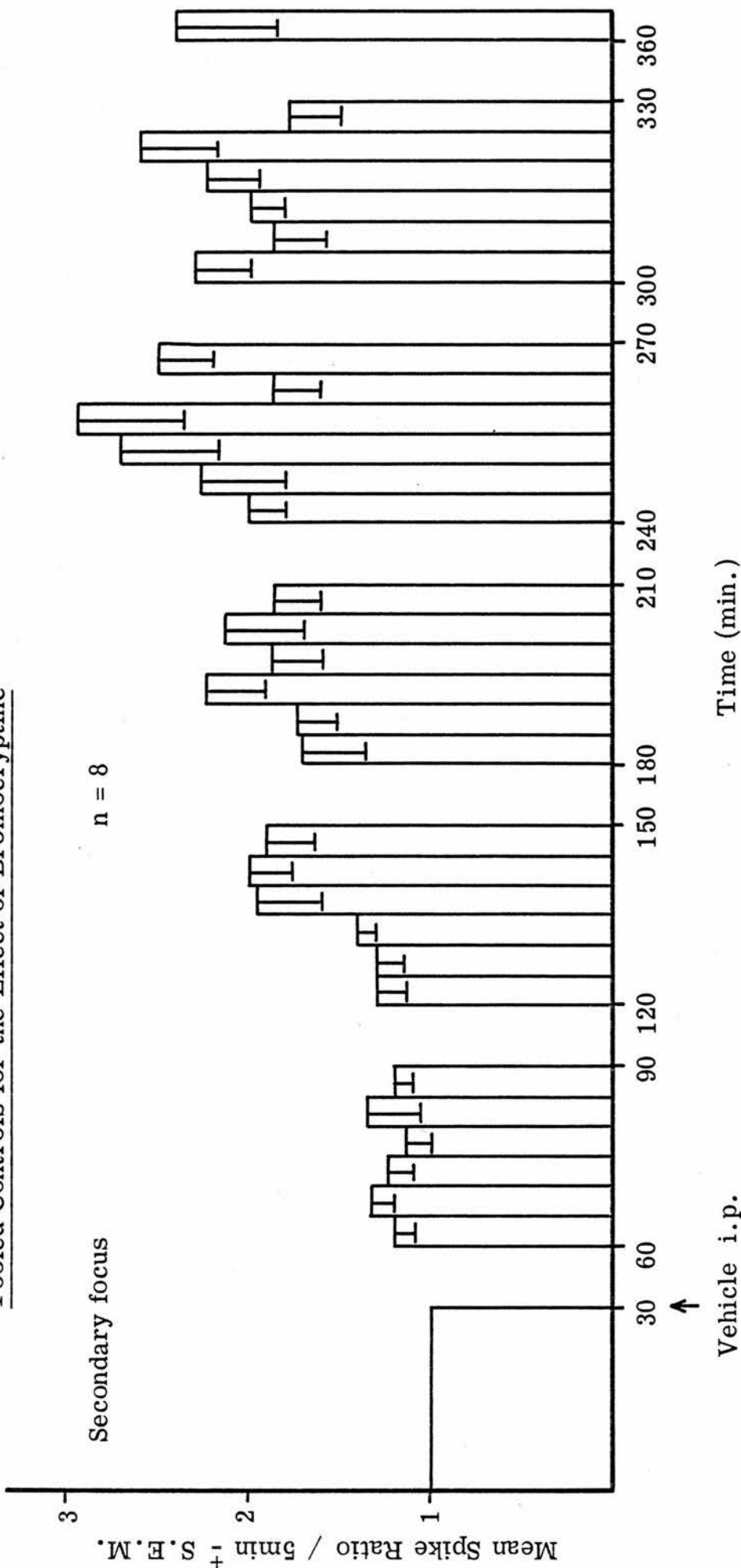


Fig 16C2

Pooled Controls for the Effect of Bromocryptine



and secondary foci which was evident within 5 min of its administration and lasted for 50, 75, 100 and 115 min with doses of 0.1, 0.25, 0.5 and 1 mg/kg, respectively (Mann-Whitney-U-Test). Using Kruskal-Wallis one-way analysis of variance, this effect was found to be dose-dependent.

For primary focus  $H = 18.7, P < 0.001$

For secondary focus  $H = 20.5, P < 0.001$ .

This effect was also associated with reduction in epileptic forelimb and whisker twitches, together with signs of dopamine receptor stimulation in the form of increased locomotion, arousal and stereotyped behaviour.

The saline-injected controls displayed an increase in spike activity in both primary one and primary two. Their motor epileptic manifestations were marked, but showed no other behaviour changes.

#### 4. Effect of CF 25-397 on epileptic activity

(See Figs. 18a, b, c, d, e). CF 25-397 had no effect on epileptic activity with a dose as high as 20 mg/kg, i.p. However, with much higher dose of 40 mg/kg, i.p. this drug induced a significant reduction in spike activity of cortical foci within 1 hr of its administration (Mann-Whitney-U-Test), as well as in the motor epileptic manifestations. On behaviour, arousal was increased, locomotion was mildly increased, but stereotyped behaviour was not seen as with apomorphine, lisuride or bromocryptine.

In general, the effect of CF 25 tended to be milder and of

Legend to Fig. 17

EFFECT OF LISURIDE HYDROGEN MALEATE ON EPILEPTIC ACTIVITY  
IN COBALT-IMPLANTED RATS.

n = number of rats

- Each bar represents the mean spike ratio per 5 min  $\pm$  standard error (S.E.M.).

1. ECoG recording was done for a control period of 30 min. The average computed spike count per 5 min during that period from either the primary or secondary focus, was represented as a ratio of 1.

2. Lisuride was injected intraperitoneally at 30 min.

Fig. 16A : 0.1 mg/kg lisuride was injected  
B : 0.25 mg/kg lisuride was injected  
C : 0.5 mg/kg lisuride was injected  
D : 1 mg/kg lisuride was injected  
E : Equal volume of saline was injected into controls (pooled results).

3. ECoG recording was continued for 2 hrs after the injection.

During this period of each animal, each spike ratio per 5 min represents the computed spike count per 5 min from either the primary or secondary focus expressed as a fraction of average count per 5 min during the initial control period of 30 min.

4. Comparison of effect between drug-treated and saline-injected animals was done with the Mann-Whitney-U-test.

\*  $P < 0.002$   
+  $P = 0.002 - 0.02$   
‡  $P = 0.02$   
‡†  $P = 0.02 - 0.05$   
‡‡  $P = 0.05$

5. Kruskal-Wallis one-way analysis of variance showed the effect of lisuride to be dose-dependent ( $P < 0.001$ ).



Fig 17A

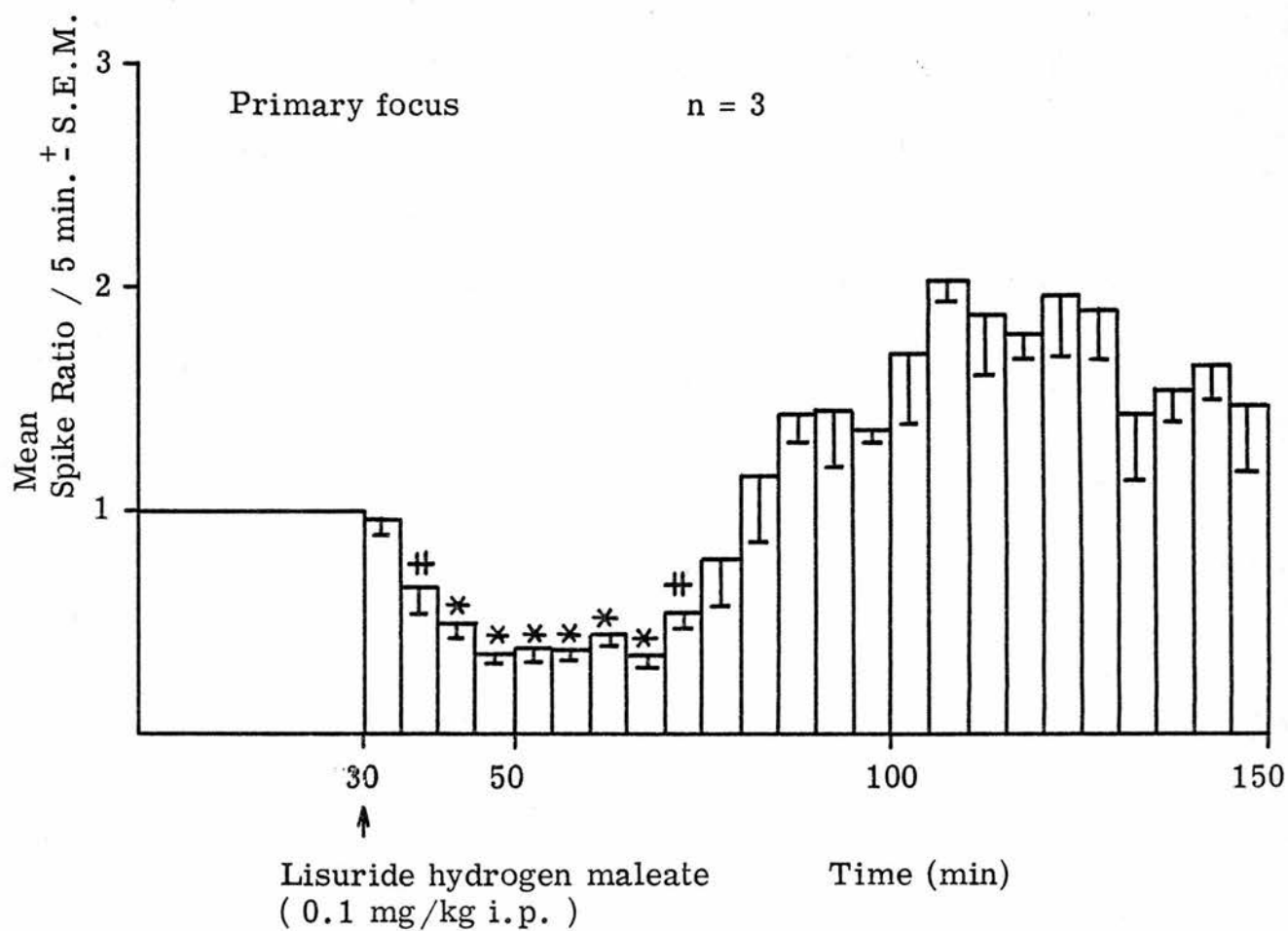
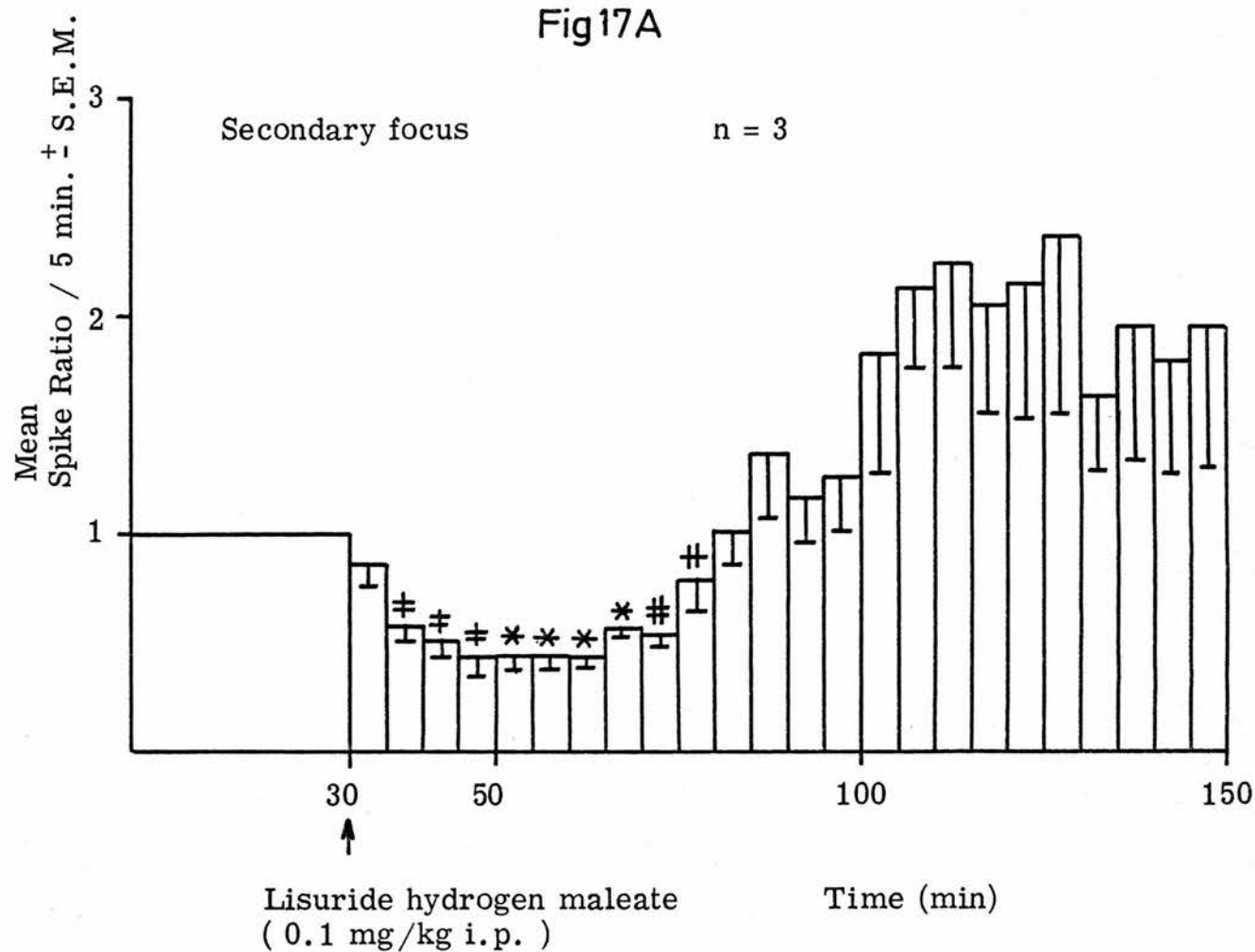


Fig 17 B

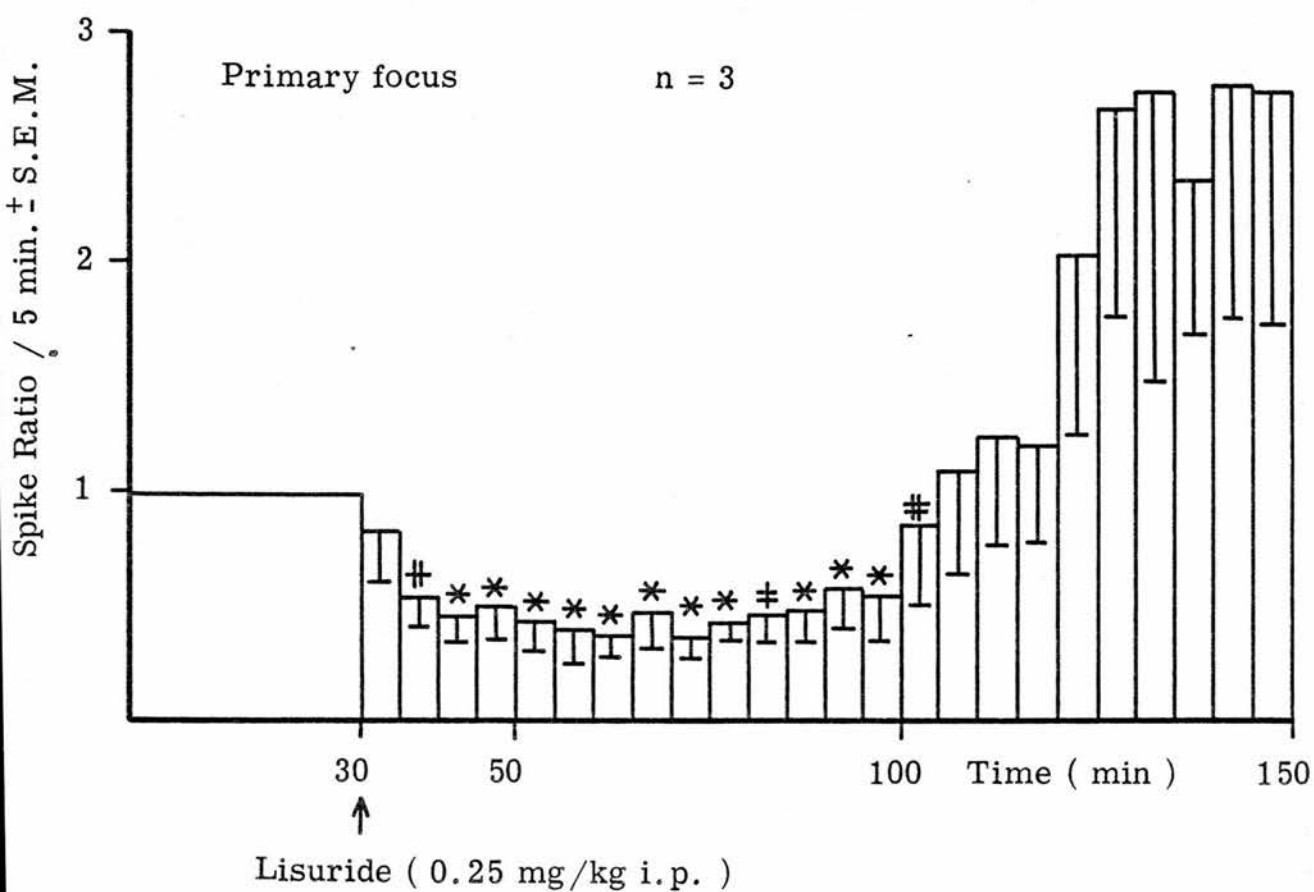
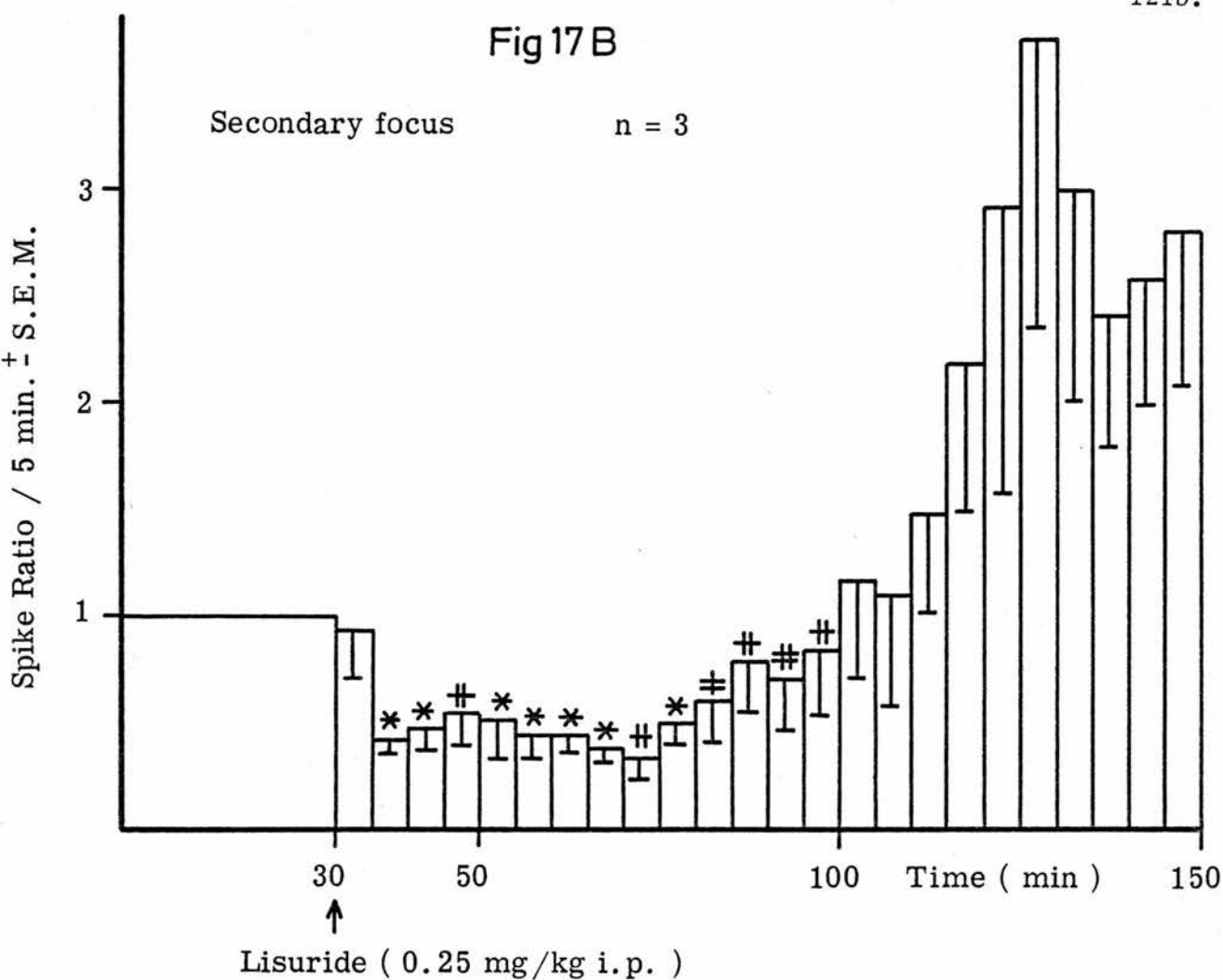


Fig 17C

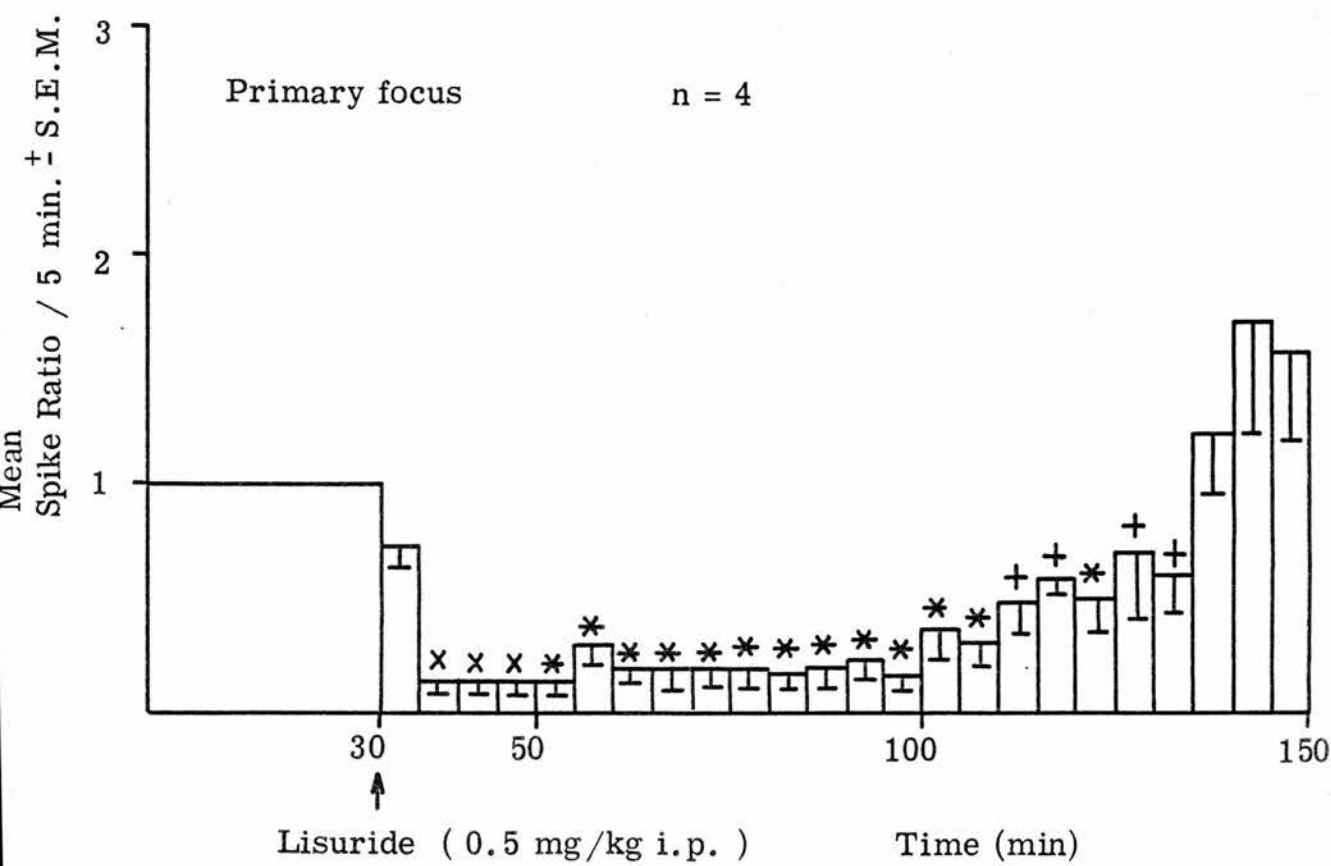
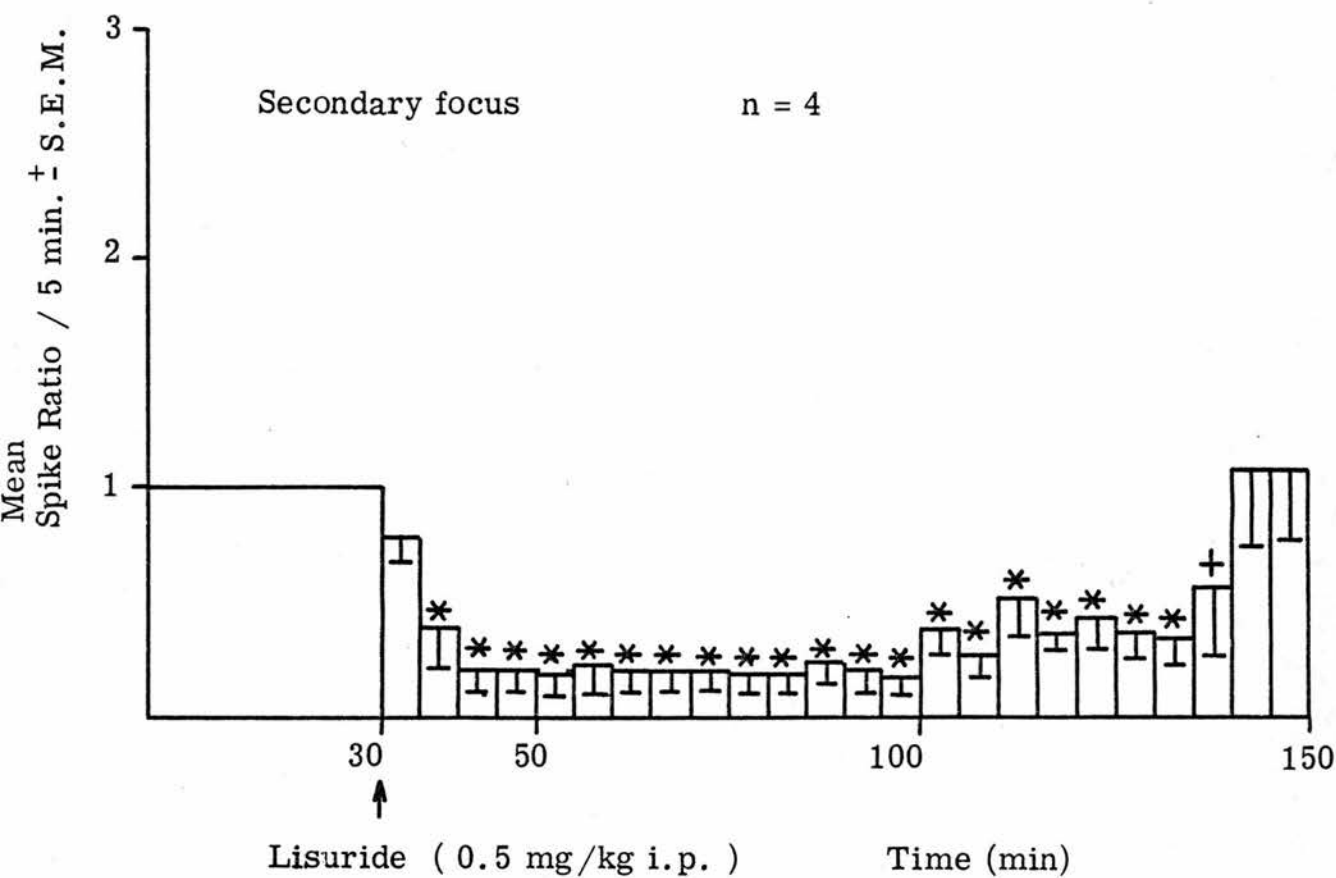


Fig17D

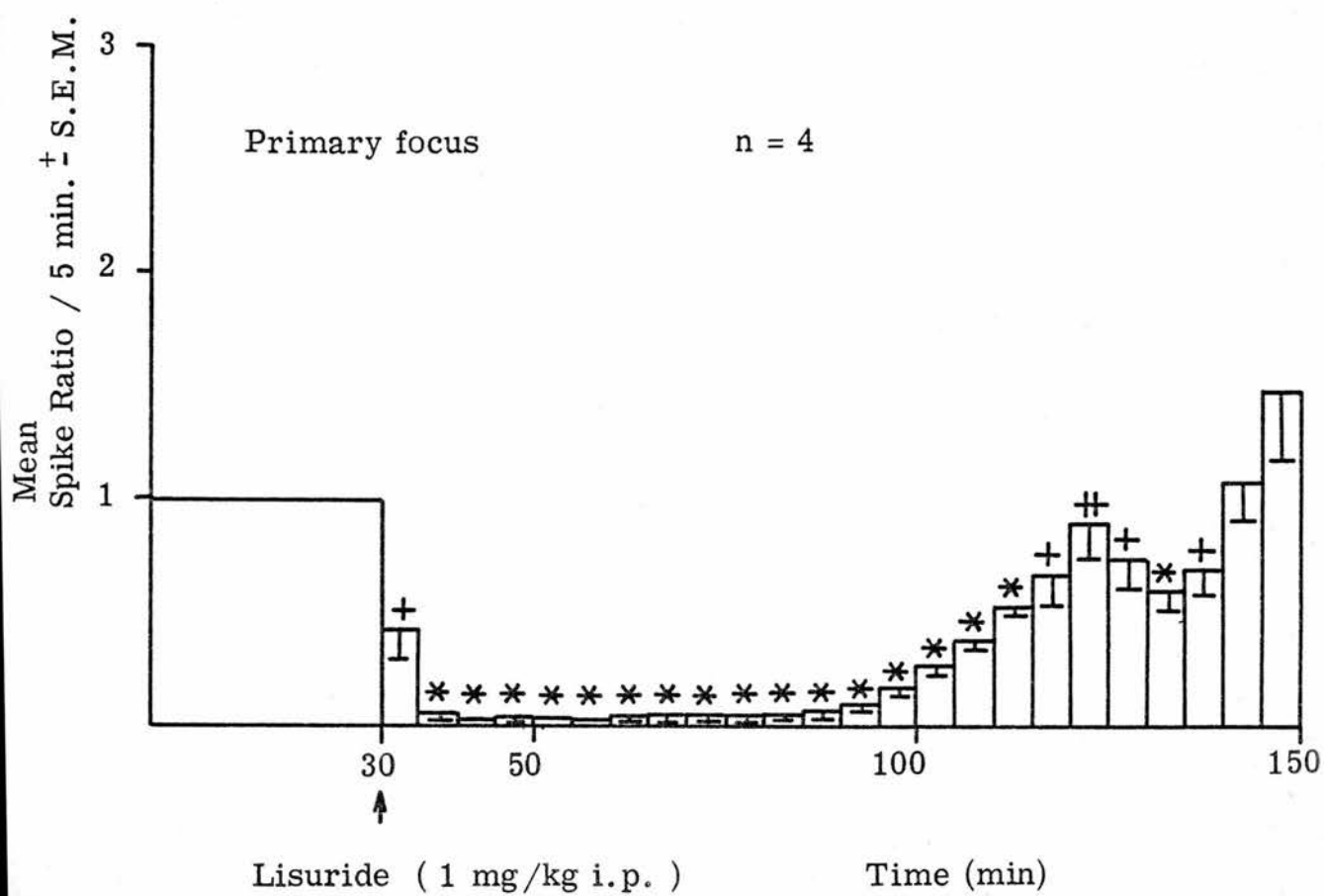
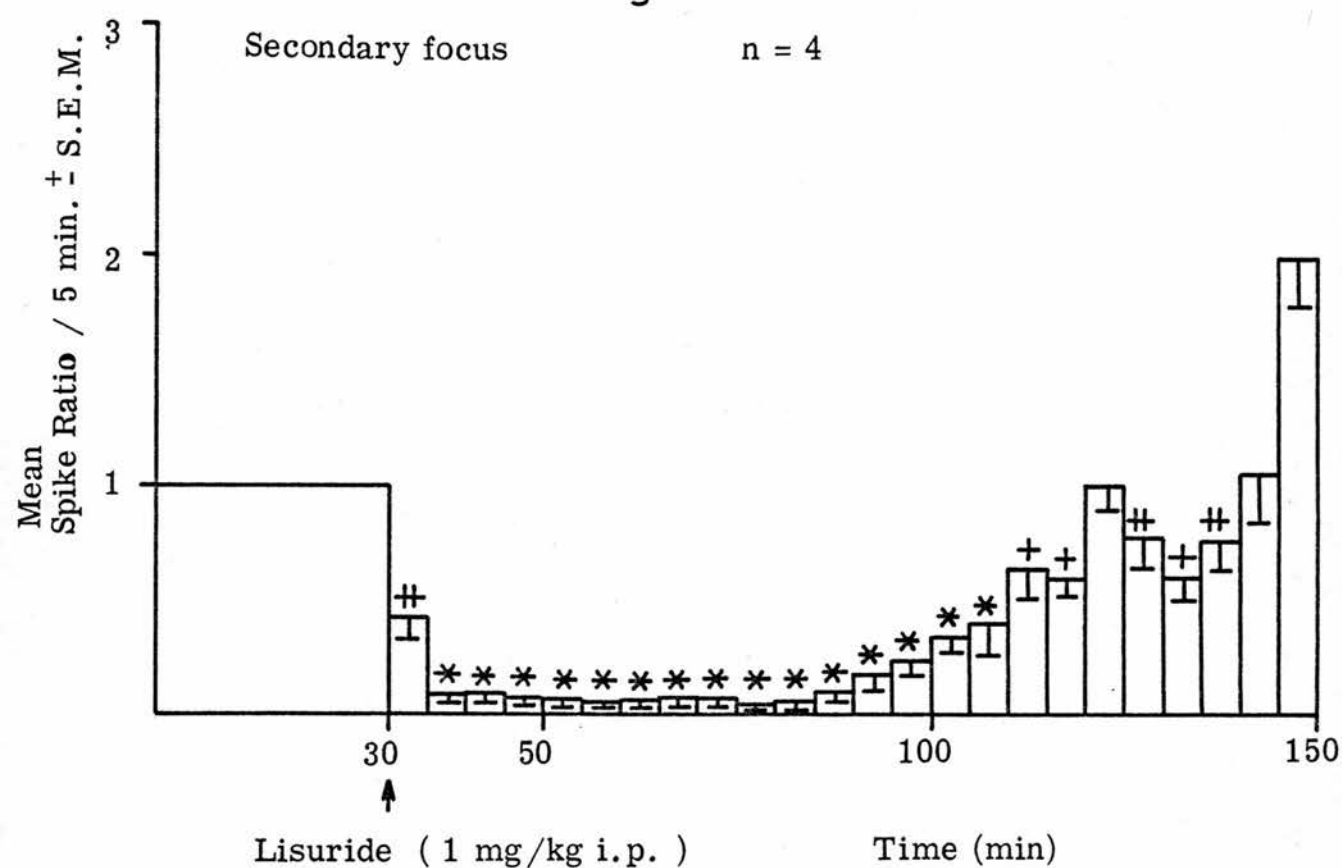
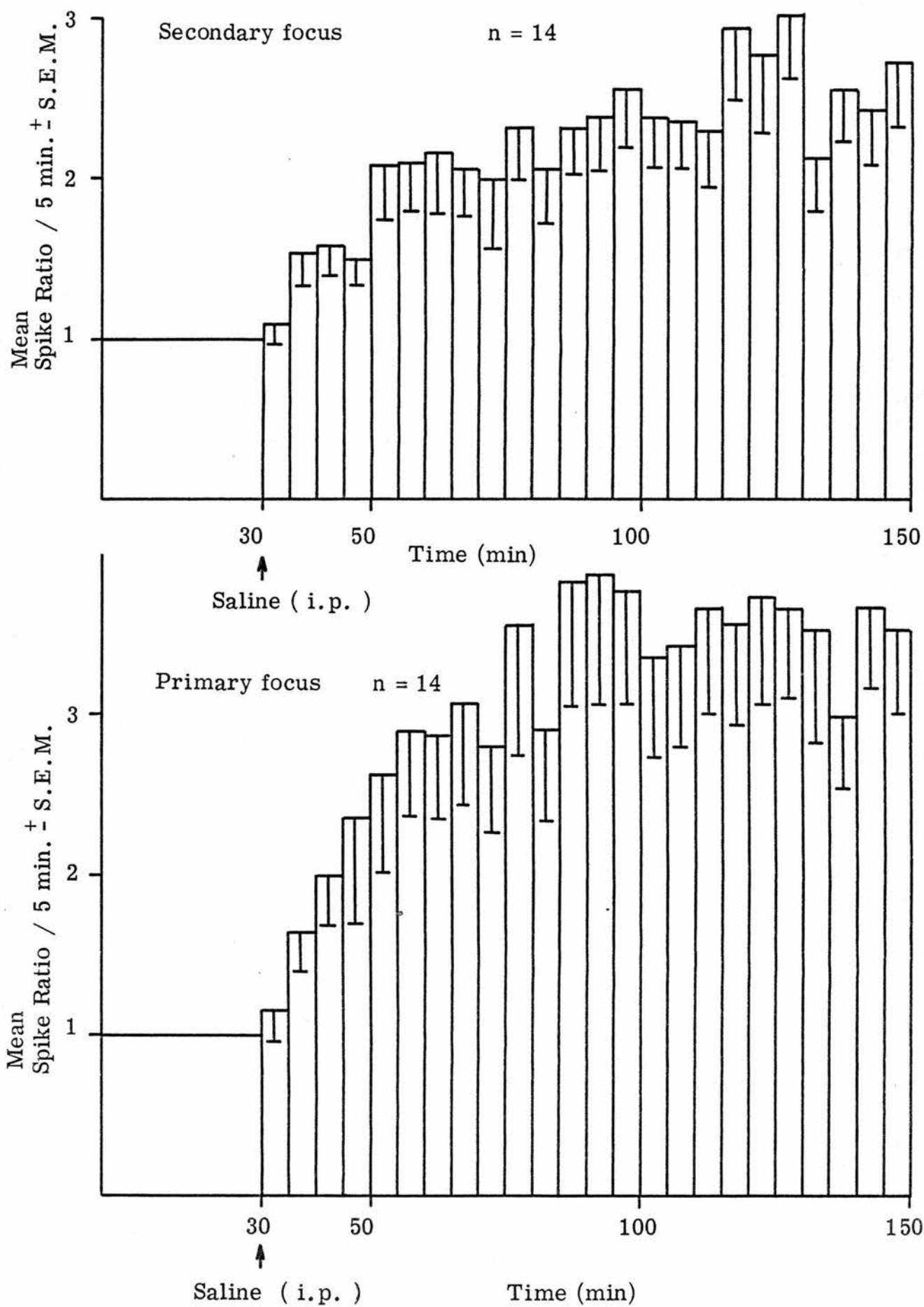


Fig 17E



Legend to Fig. 18

EFFECT OF CF 25-397 ON EPILEPTIC ACTIVITY.

n = number of animals

- Each bar represents the mean spike ratio per 5 min  $\pm$  standard error (S.E.M.).

1. ECoG recording was done for an initial period of 30 min. The average computed spike count per 5 min during that period from either the primary or secondary focus was represented as a ratio of 1.

2. CF 25-397 was injected intraperitoneally at 30 min.

Fig. 18A : 20 mg/kg CF 25-397 was injected  
B : 40 mg/kg CF 25-397 was injected  
C : Equal volume of vehicle was injected  
into controls (pooled results).

3. Recording was continued every second half hour for the following 6 hrs after the injection.

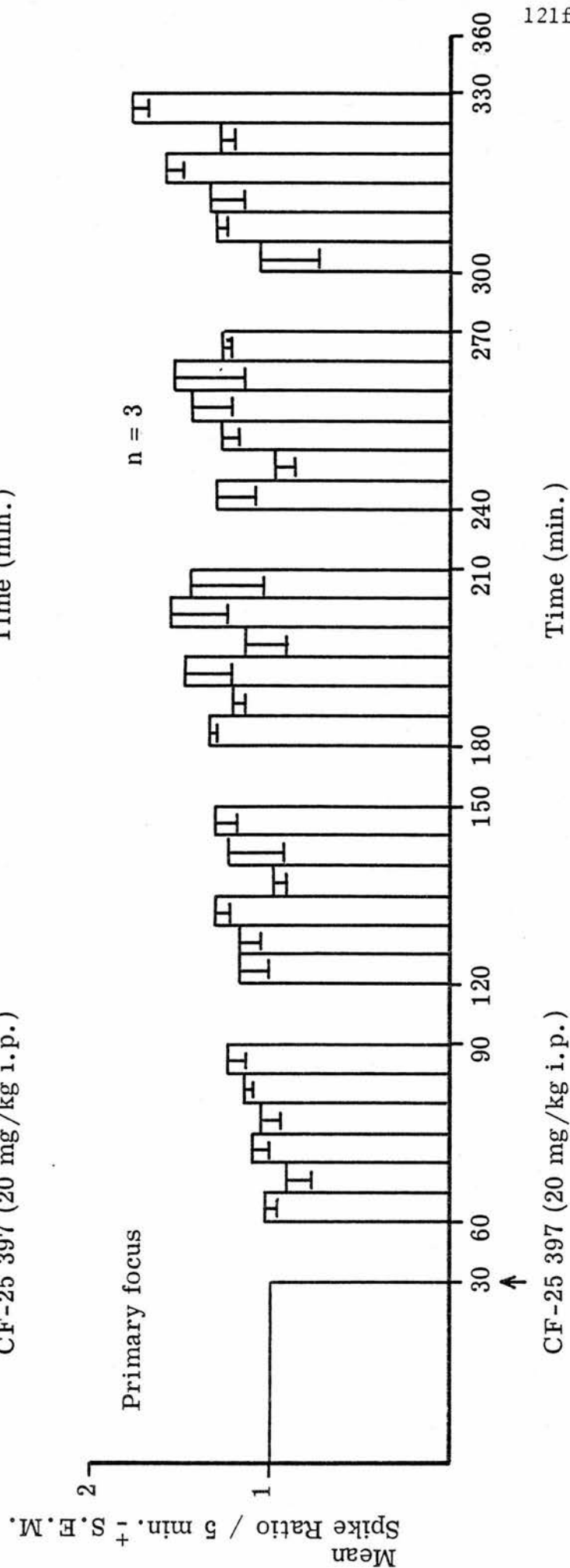
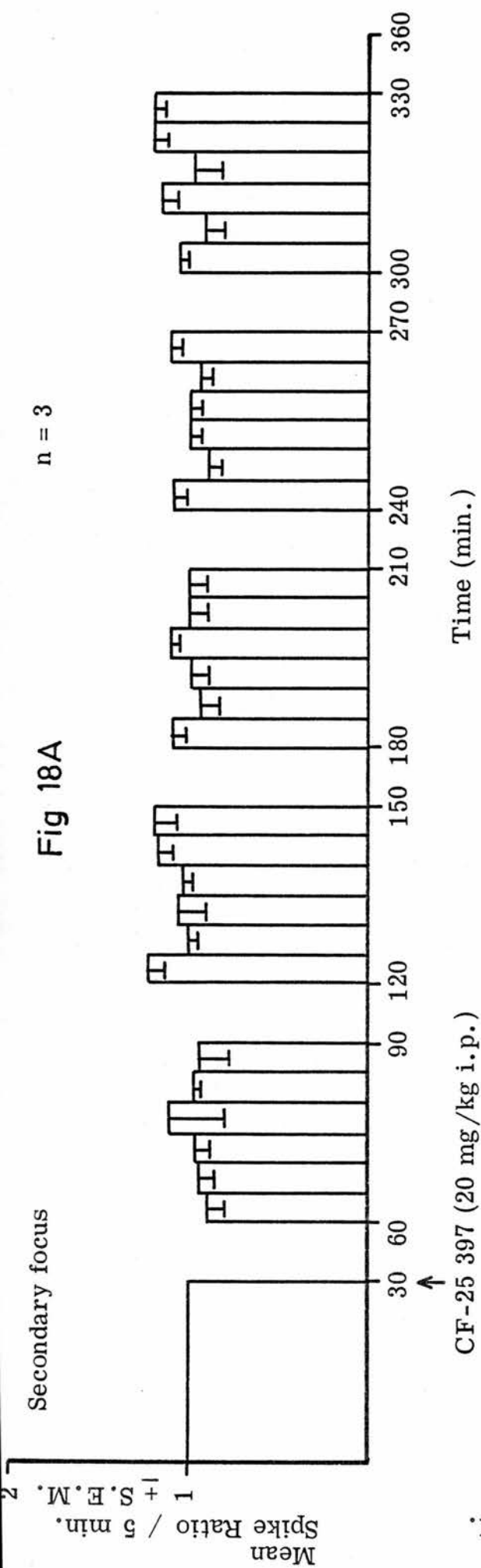
During the post-injection period of each animal, each spike ratio per 5 min was determined as described before.

4. Comparison between drug-treated and control rats was done with the Mann-Whitney-U-test.

\* P = 0.006  
+ P = 0.012  
‡ P = 0.024  
† P = 0.042

n = 3

Fig 18A

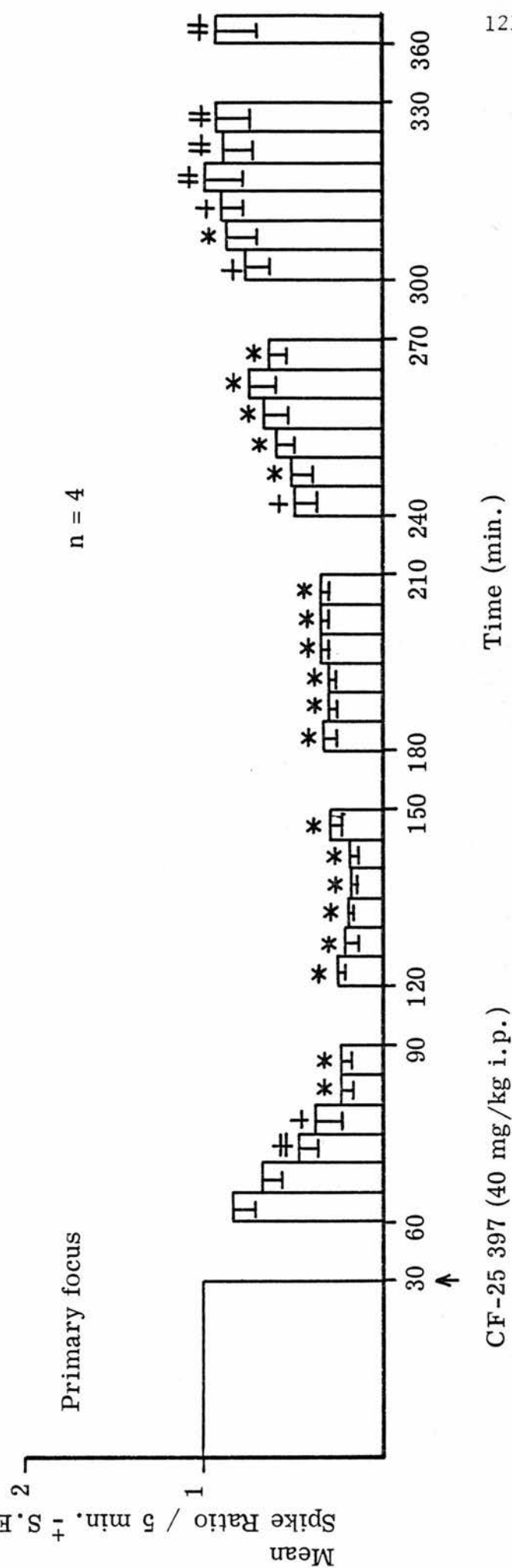


n = 4

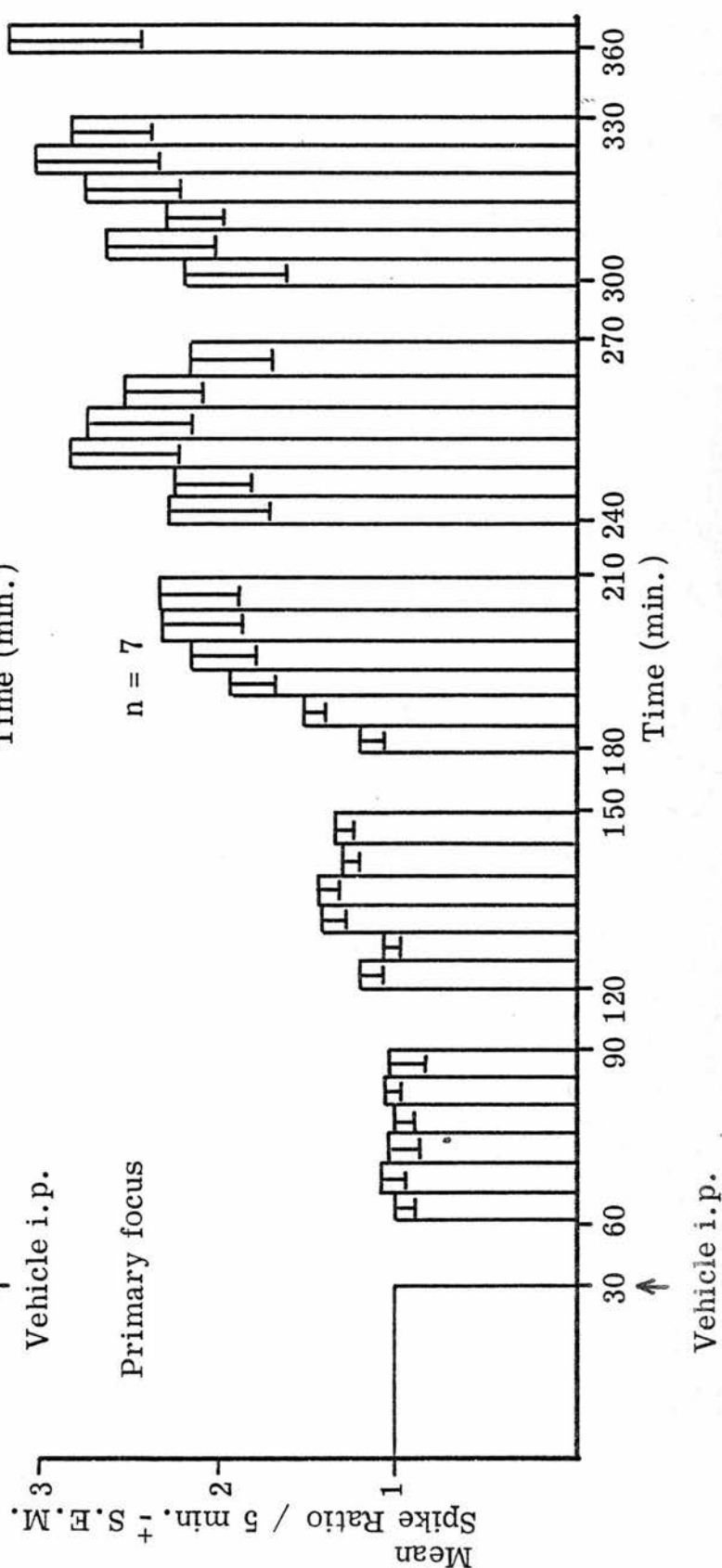
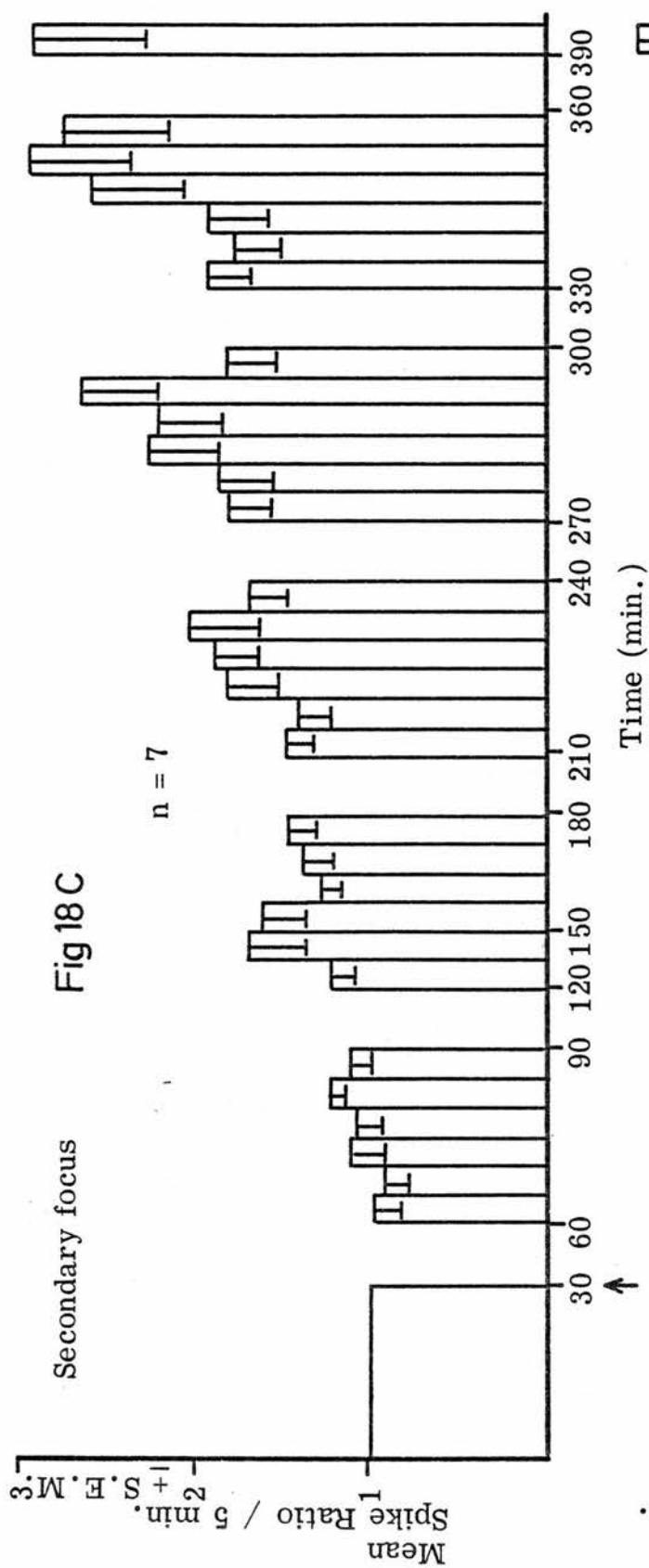
Fig 18B



n = 4







shorter duration than that of bromocryptine. Some animals seemed to recover after 7 hrs. However, each animal was back to normal by 24 hrs after its injection. The vehicle-treated rats showed no significant alteration in epileptic activity or behaviour.

B. EFFECT OF DAILY ADMINISTRATION OF BROMOCRYPTINE (20 mg/kg i.p.) ON THE DEVELOPMENT OF EPILEPTIC ACTIVITY IN COBALT-IMPLANTED RATS

(See Figs. 19a, b).

The chronic administration of bromocryptine did not prevent the appearance of primary and secondary foci in cobalt-implanted rats. The foci appeared at the same time in comparison to controls. The early stages of development of primary and secondary foci were not significantly different from controls. However, after 5-6 days the spike activity of the cortical foci in the drug-treated rats were significantly lower in comparison to controls, and persisted to be significantly lower until the 12th day of observation (Kruskal-Wallis one-way analysis of variance).

The secondary focus seemed to be more markedly suppressed than the primary focus by chronic bromocryptine treatment.

C. EXPERIMENTS WITH THE DOPAMINE-RECEPTOR ANTAGONIST, PIMOZIDE

1. Effect of pimozide on epileptic activity

(See Figs. 20a, b). Pimozide, 1 mg/kg, i.p., induced a significant increase in epileptic activity on both primary and secondary foci which was evident for 10-15 min of its injection (Mann-Whitney-U-Test), and lasted at least 2 hrs after the injection. The vehicle-treated controls showed no significant changes in epileptic activity or behaviour.

Legend to Fig. 19

EFFECT OF CHRONIC ADMINISTRATION OF BROMOCRYPTINE ON THE DEVELOPMENT OF EPILEPTIC FOCI IN COBALT-IMPLANTED RATS.

n = number of animals

- Each bar represents the mean spike count per min  $\pm$  standard error (S.E.M.).

1. Bromocryptine (20 mg/kg) was injected daily, i.p., into one group of rats starting on the day of cobalt implantation. Control rats received an equivalent volume of vehicle, similarly.
2. Daily 10 min ECoG recording was done from both groups of rats before the injection.
3. Comparison between drug-treated and control rats were done using the Kruskal-Wallis analysis of variance.

\* P = 0.01 - 0.01

+ P = 0.01 - 0.02

# P = 0.02 - 0.05

Fig 19A

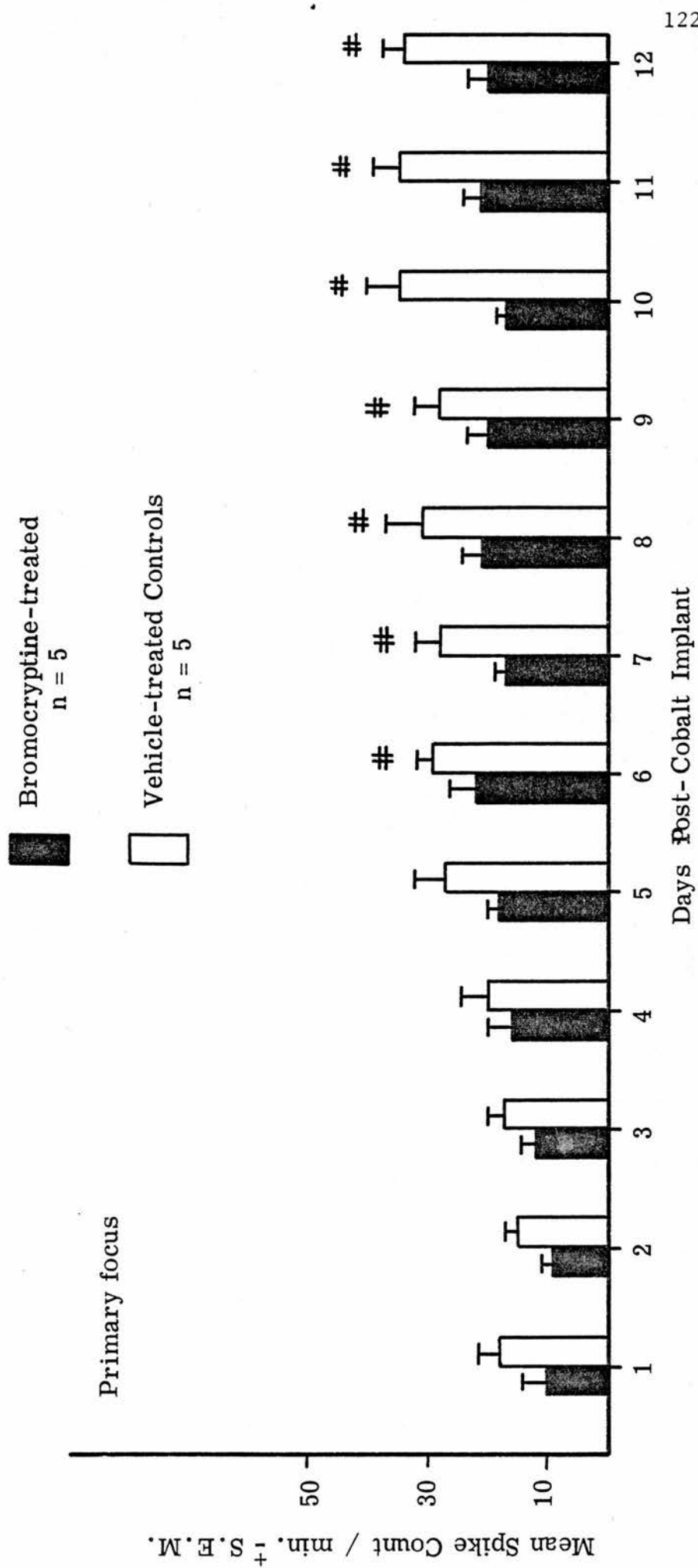
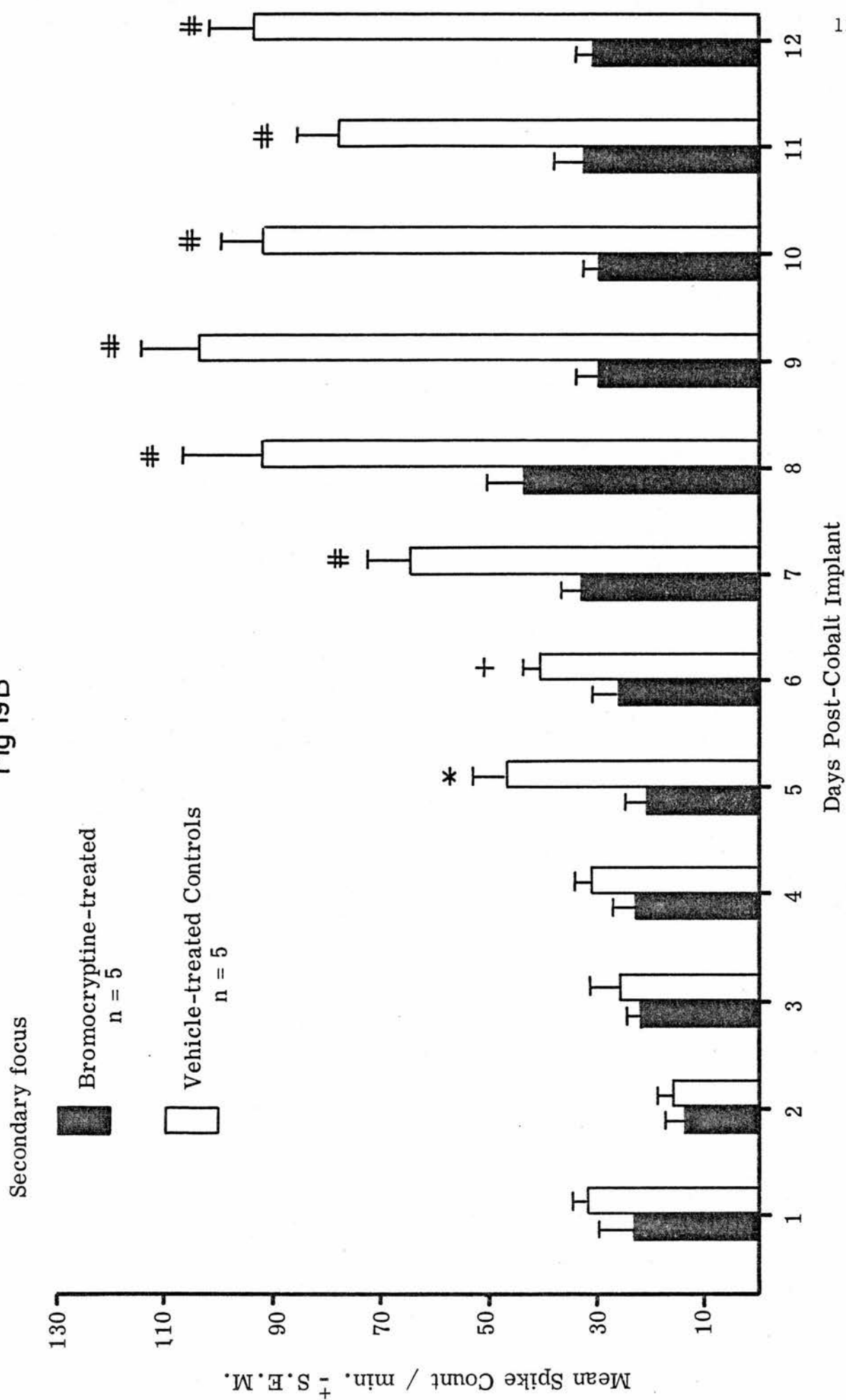


Fig 19B



2. Effect of pimozide on the spike suppressing effect of apomorphine, lisuride, bromocryptine and CF 25.

(See Figs. 21a and b, Figs. 22a and b, Figs. 23a and b, Figs 24a and b, respectively). Pretreatment with pimozide (1 mg/kg, i.p.) significantly prevented the effect of these dopamine agonists on epileptic activity of both primary and secondary foci (Mann-Whitney-U-Test), as well as the behavioural manifestation induced by these drugs.

D. EXPERIMENT ON THE STRIATUM IN COBALT-INDUCED EPILEPSY

1. Effect of dopamine

The bilateral injection of dopamine (25  $\mu$ g into each striatum) in cobalt-induced epileptic rats produced a significant reduction in spike activity of both primary and secondary foci (Mann-Whitney-U-Test) which was evident within 5 min of injection and lasted for 55 min (Figs. 25a and b). This effect was associated with suppression of whiskers and forelimb twitches, as well as the appearance of signs of dopaminergic stimulation of the striatum such as increased arousal, locomotion and stereotyped behaviour.

Furthermore, the unilateral injection of dopamine (25  $\mu$ g) into the right striatum (Figs. 26a and b) reduced significantly the epileptic activity of both primary and secondary foci (Mann-Whitney-U-Test), and affected as well a reduction in the motor epileptic manifestations. This effect was associated with increased locomotion and turning of the animal towards the left side, i.e. contralateral to the side of injection. This effect lasted for about 40 min before disappearing.

Legend to Fig. 20

EFFECT OF PIMOZIDE ON EPILEPTIC ACTIVITY.

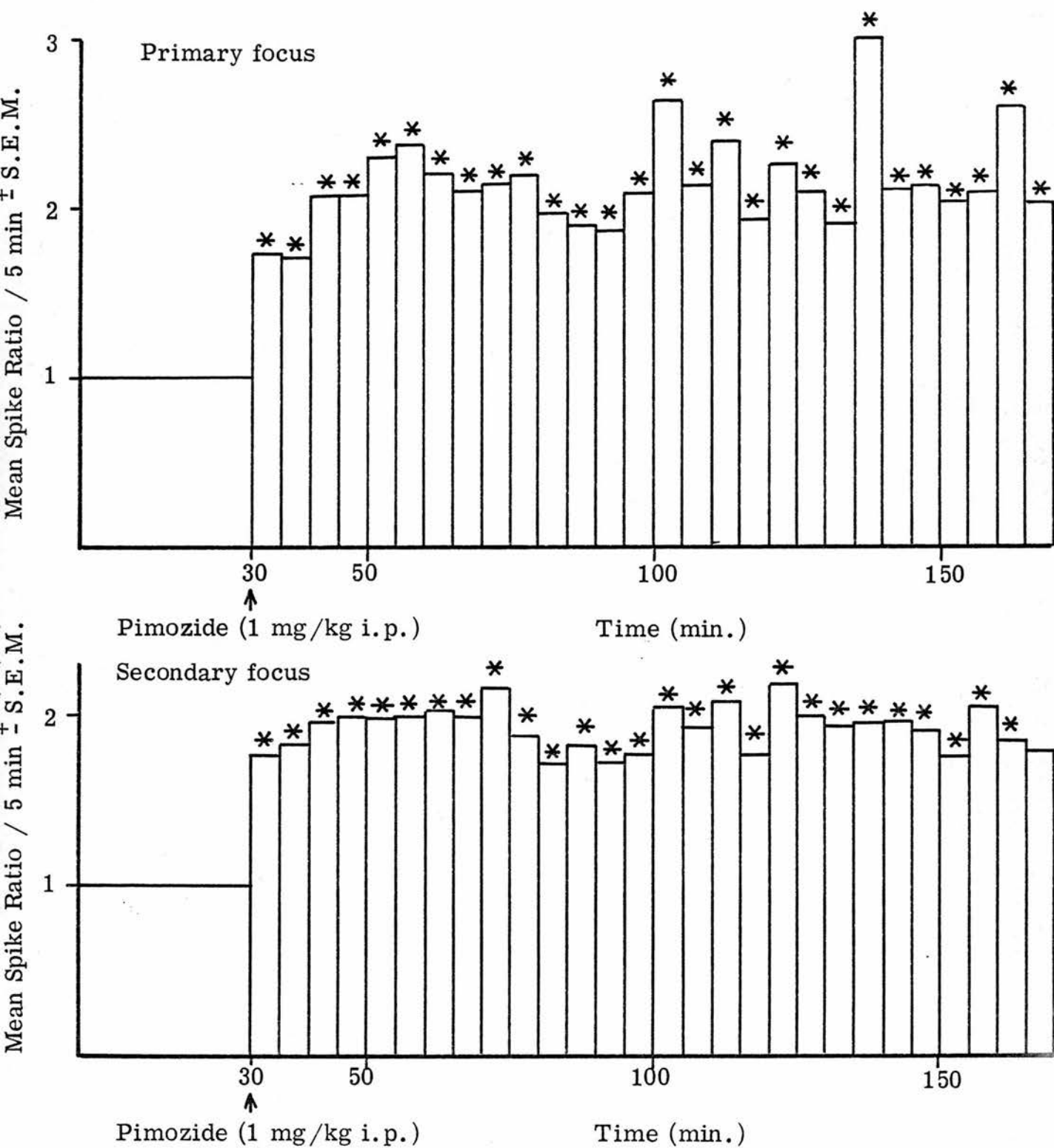
n = number of animals.

- Each bar represents the mean spike ratio per 5 min  $\pm$  standard error (S.E.M.).

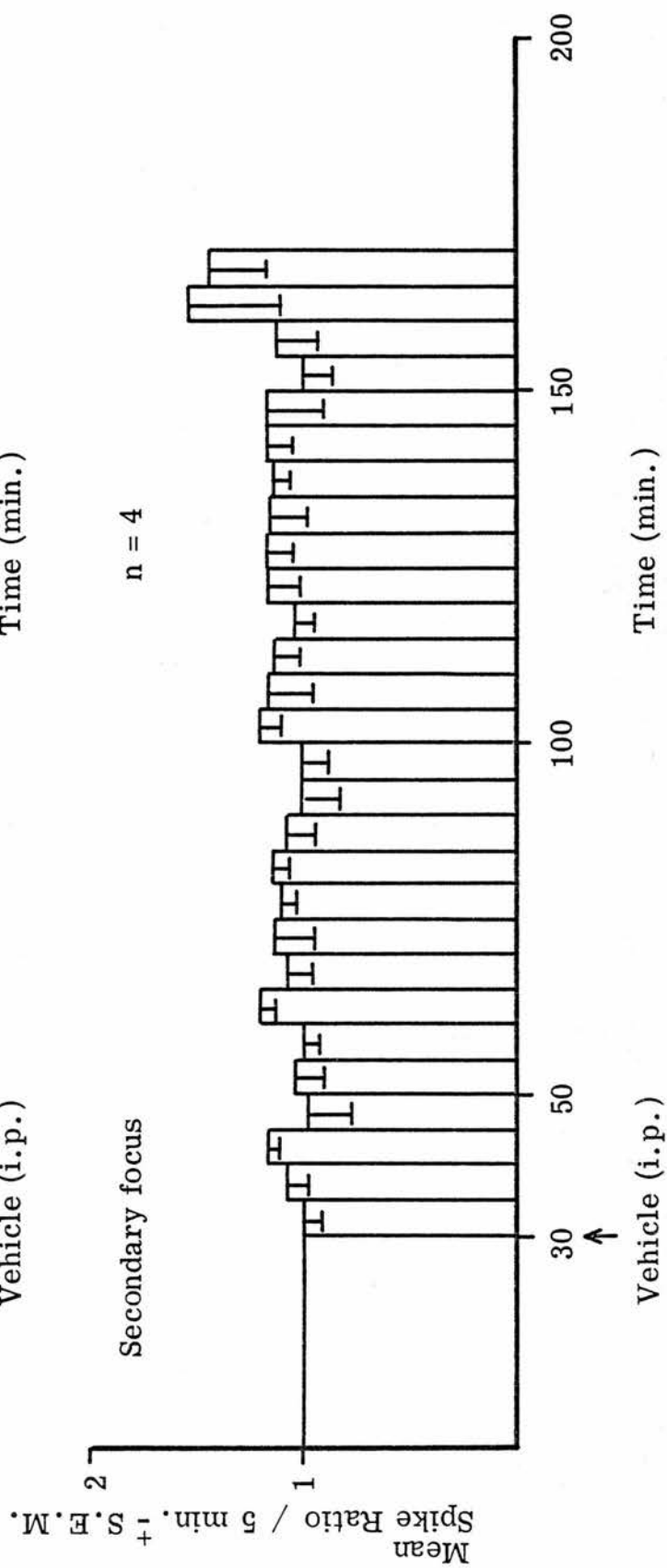
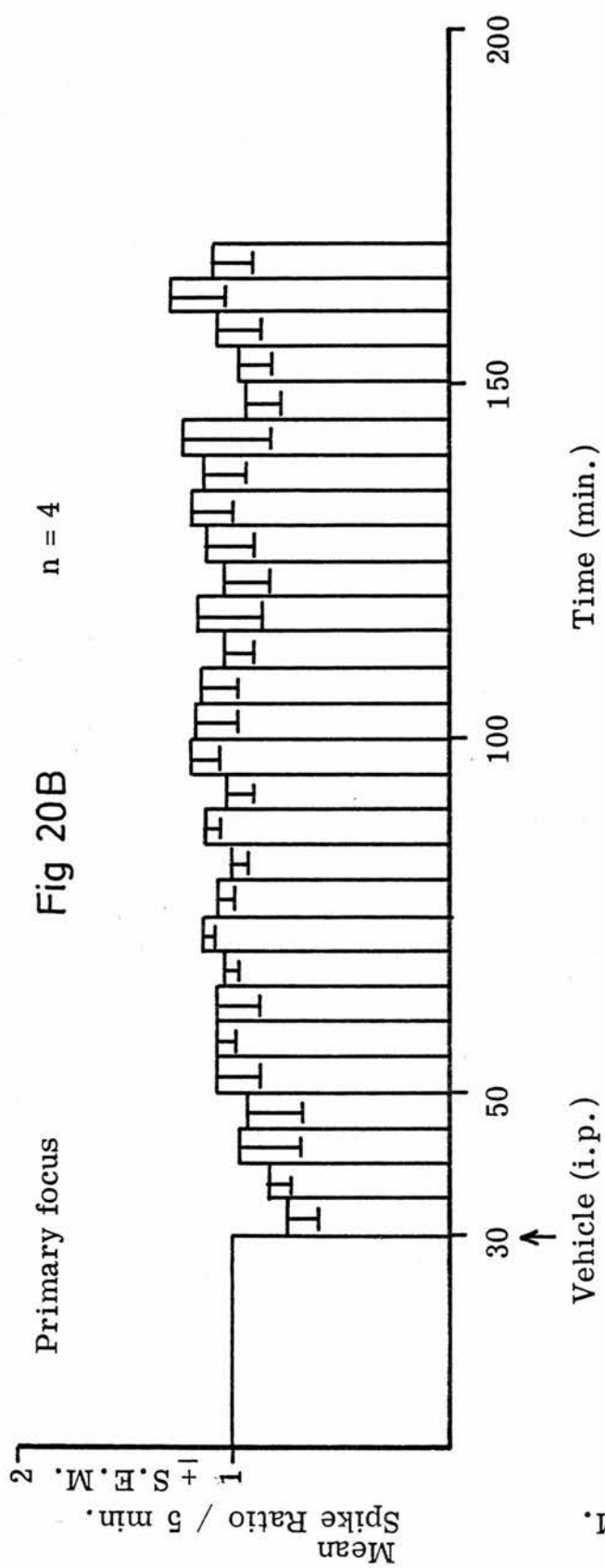
1. ECoG recording was done for a control period of 30 min. The average computed spike count per 5 min during that period in either the primary or secondary focus was expressed as a ratio of 1.
2. Pimozide (1 mg/kg) was injected intraperitoneally at 30 min. Control rats received an equivalent volume of vehicle.
3. Recording was continued for 2 hrs after the injection.  
  
During the post-injection period of each animal, the spike ratio/5 min was determined as a ratio of the computed spike count per 5 min from either focus to its average count per 5 min during the initial control period of 30 min.
4. Comparison between drug-treated and vehicle-treated animals was done using the Mann-Whitney-U-test.

\* P = 0.05.

Fig 20 A







Legend to Fig. 21

EFFECT OF PIMOZIDE ON APOMORPHINE-INDUCED SUPPRESSION OF  
EPILEPTIC ACTIVITY.

n = number of rats.

- Each bar represents the mean spike ratio per 5 min  
± standard error (S.E.M.).

1. ECoG recording was done for a control period of 30 min.  
The mean spike count per 5 min during that period from  
either the primary or secondary focus was expressed as  
a ratio of 1.

2. Pimozide (1 mg/kg, i.p.) was injected at 30 min (Fig. 21A).

Control rats received an equivalent volume of saline (Fig. 21B).  
ECoG recording was continued for 45 min after pimozide.

3. Both groups of animals then received apomorphine (1 mg/kg, i.p.)  
and recording was continued for 2 hrs after the injection.

During the post-injection period of each animal each spike  
ratio per 5 min was determined as described before.

4. Comparison between pimozide-treated and vehicle-treated  
animals were done with the Mann-Whitney-U-test.

\* P = 0.014

+ P = 0.029

# P = 0.05

Fig 21A

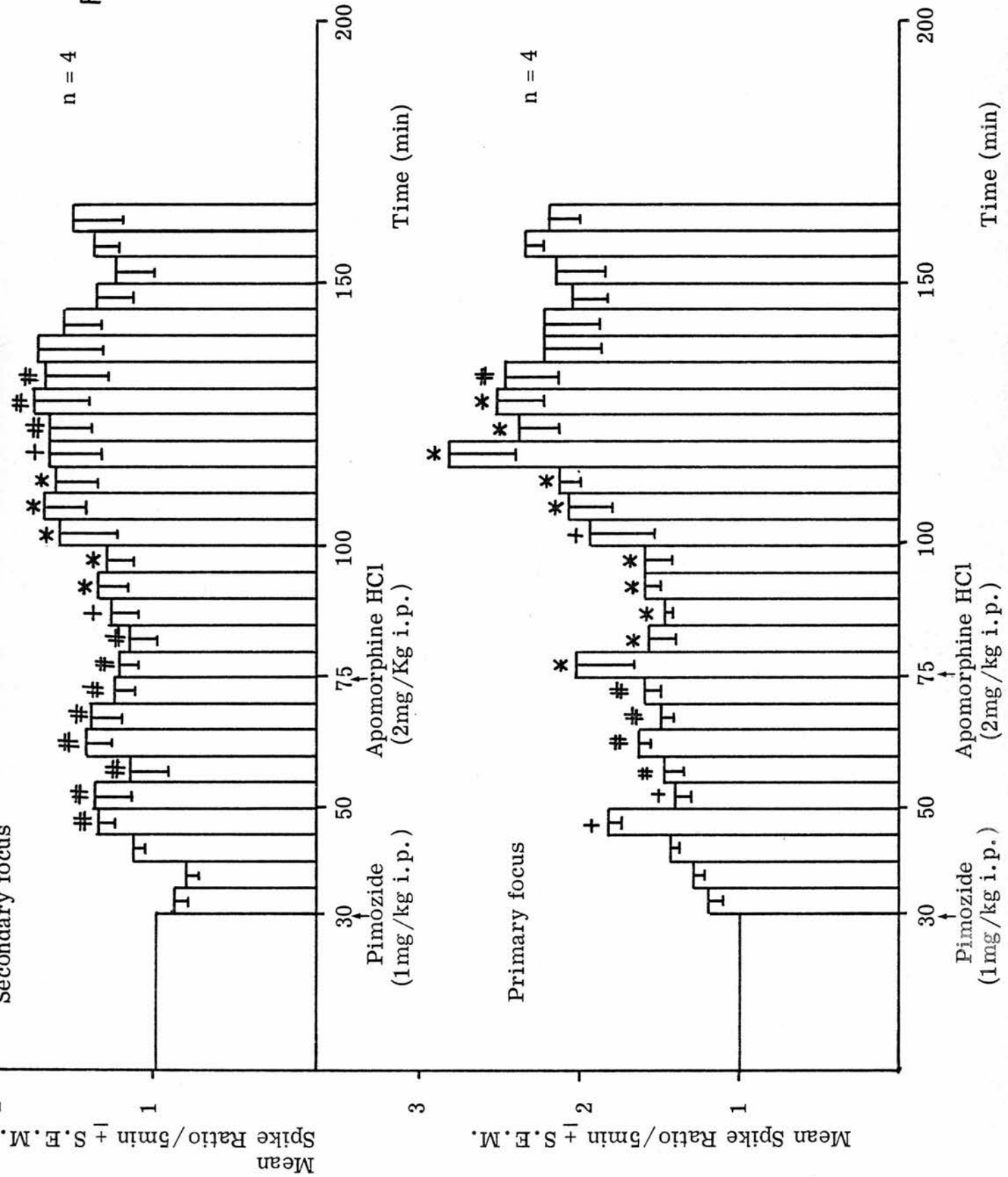
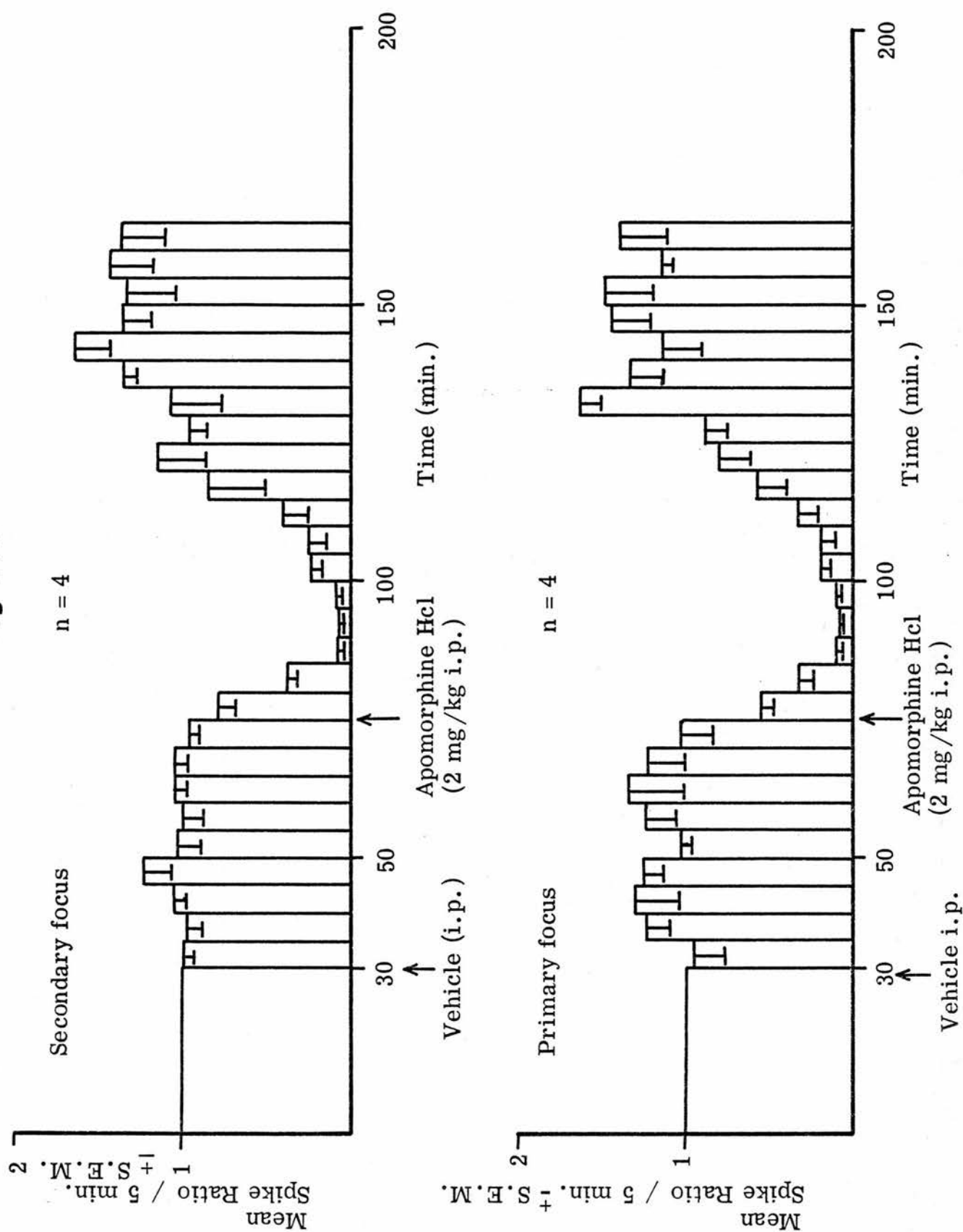


Fig 21 B



Legend to Fig. 22

EFFECT OF PIMOZIDE ON BROMOCRYPTINE-INDUCED SUPPRESSION OF  
EPILEPTIC ACTIVITY.

n = number of animals.

- Each bar represents the mean spike ratio per 5 min  
+ standard error (S.E.M.).

1. ECoG recording was done for an initial control period of 30 min. The average spike count per 5 min of either the primary or secondary focus during that period was expressed as a ratio of 1.
2. Pimozide (1 mg/kg) was injected at 30 min, i.p. (Fig. 22A). Control rats received an equivalent volume of vehicle. (Fig. 22B). Recording was continued for 20 min.
3. Both groups of animals then received bromocryptine (20 mg/kg), i.p. and recording was continued every second half hour for the following 6 hrs.

During the post-injection period of each animal, each spike ratio/5 min was determined as described before.

4. Comparison of effect between pimozide-treated and vehicle-treated animals was done with the Mann-Whitney-U-test.

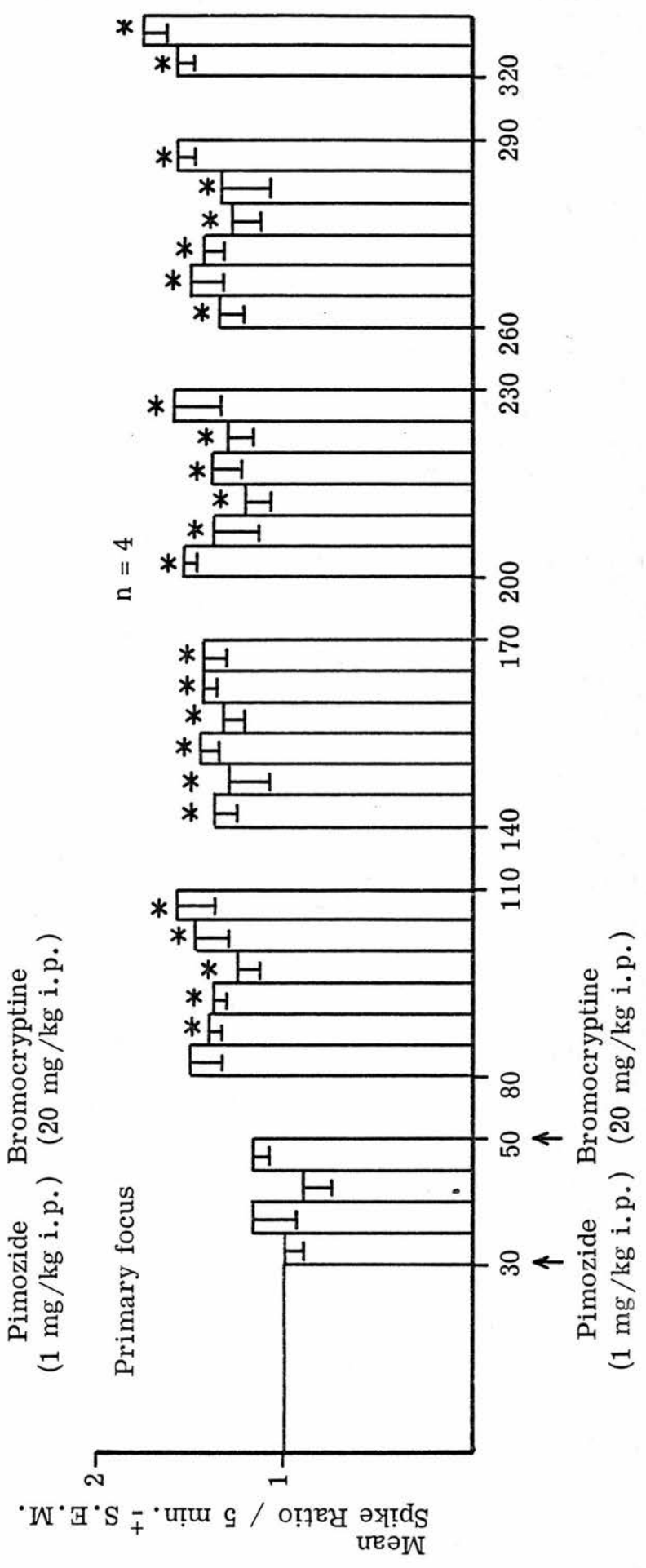
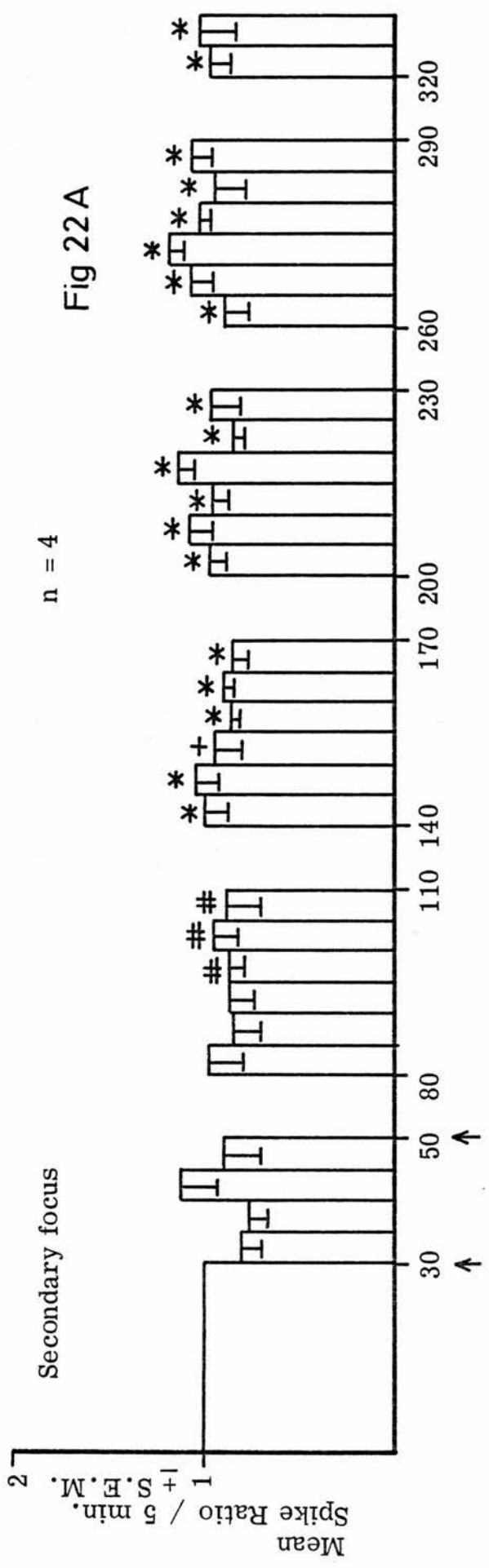
\* P = 0.014

+ P = 0.029

# P = 0.05

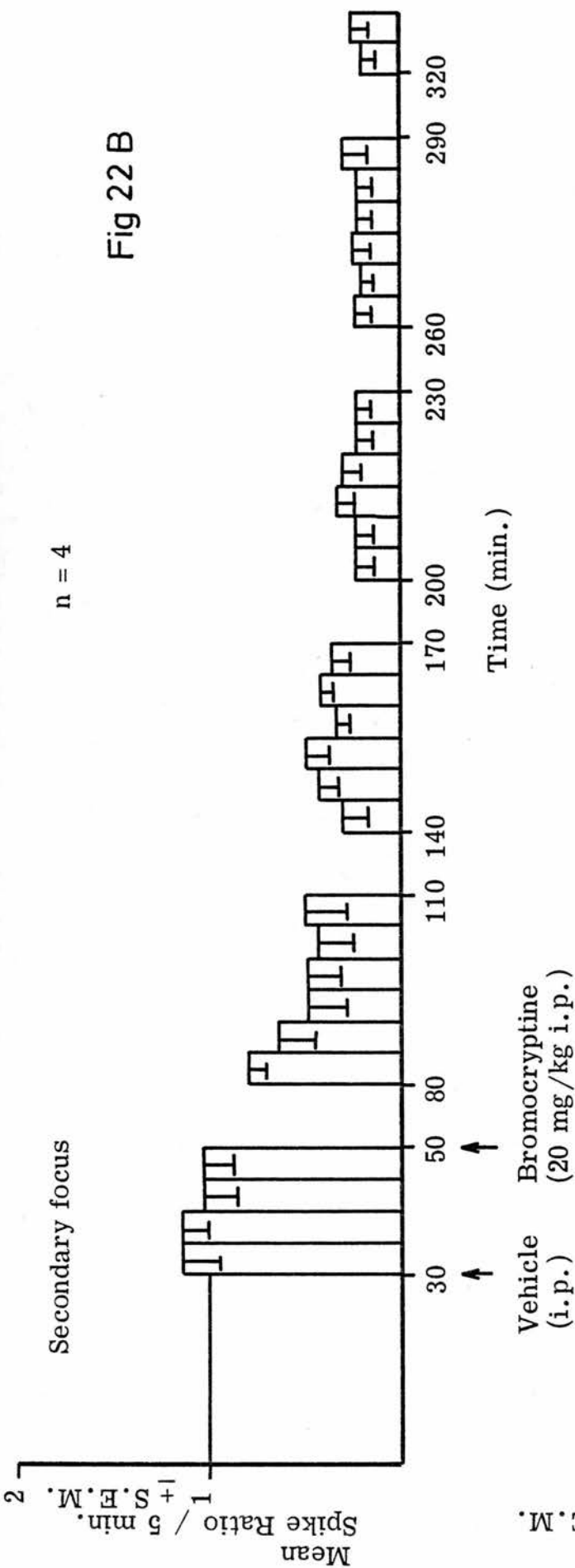
Fig 22 A

n = 4

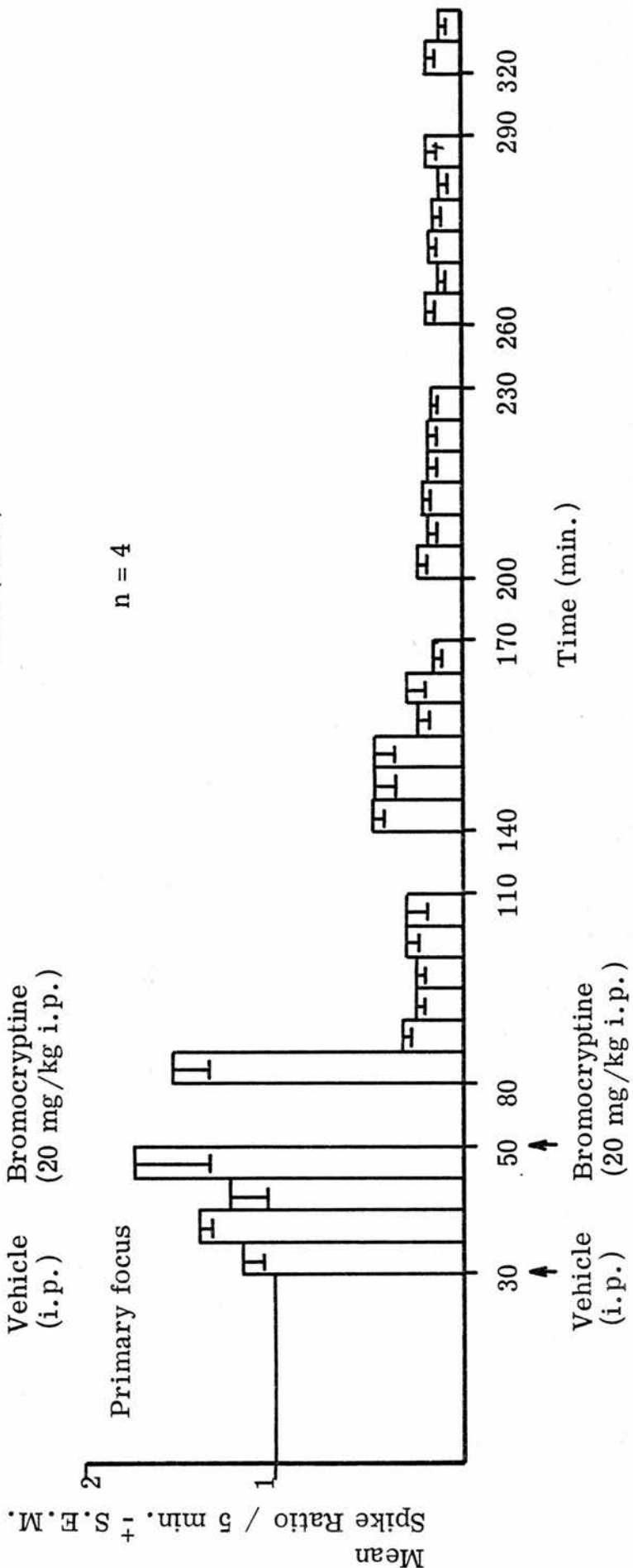


n = 4

Fig 22 B



n = 4



Legend to Fig. 23

EFFECT OF PIMOZIDE ON LISURIDE-INDUCED SUPPRESSION OF  
EPILEPTIC ACTIVITY.

n = number of animals.

- Each bar represents the mean spike ratio per 5 min  $\pm$  standard error (S.E.M.).

1. ECoG recording was done for a control period of 30 min. The mean spike count for 5 min from either the primary or secondary focus during that period was expressed as a ratio of 1.
2. Pimozide (1 mg/kg) was injected, i.p., at 30 min (Fig. 23A). Control rats received an equivalent volume of vehicle (Fig. 23B).
3. ECoG recording was continued for 45 min after injection of pimozide. Both groups of animals then received lisuride (0.1 mg/kg) i.p., and recording was continued for 2 hrs after the injection.

During the post-injection period of each animal, each spike ratio per 5 min was determined as described before.

4. Comparison of effect between drug-treated and saline-treated rats was done with the Mann-Whitney-U-test.

+ P = 0.05.



Fig 23A

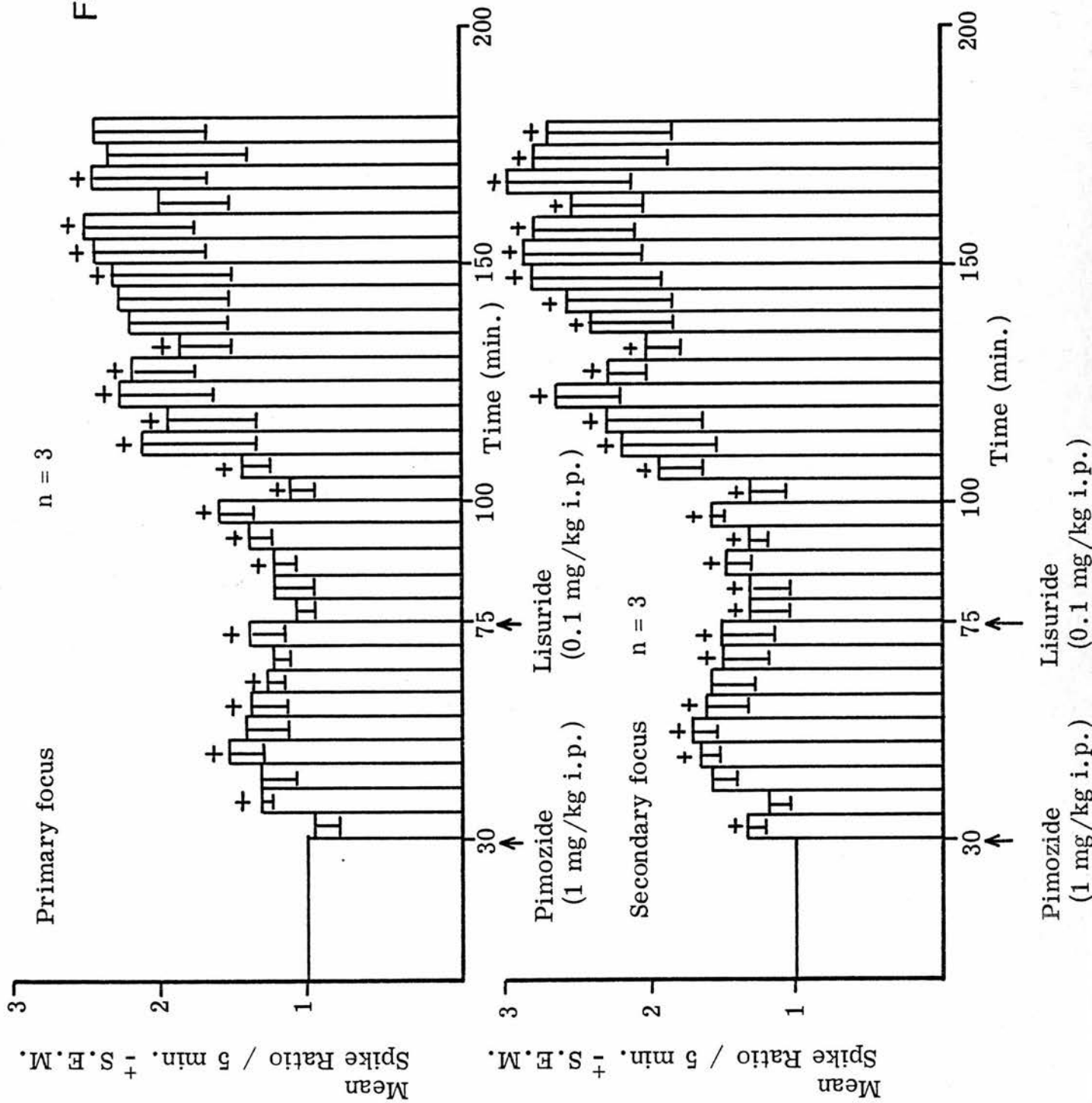
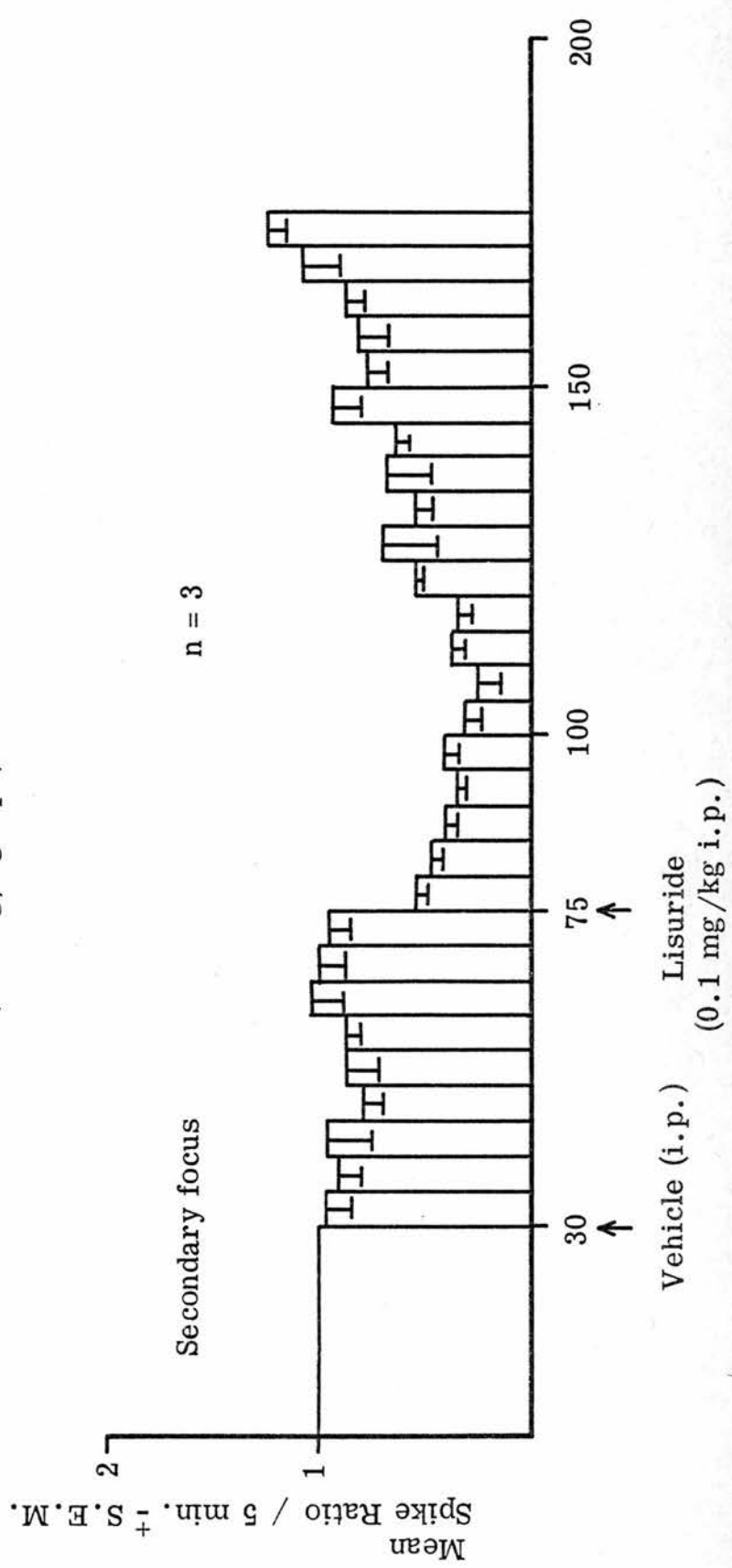
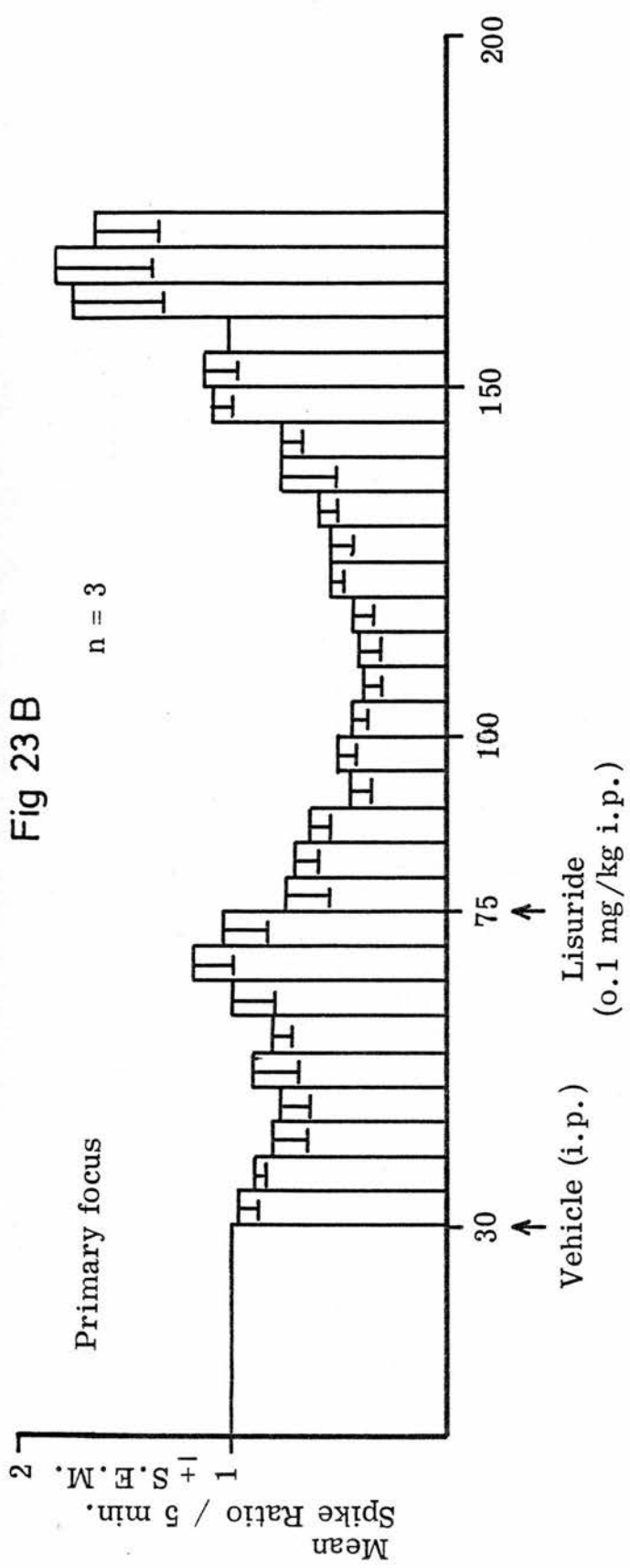


Fig 23 B



Legend to Fig. 24

EFFECT OF PIMOZIDE ON CF 25-397-INDUCED SUPPRESSION OF  
EPILEPTIC ACTIVITY.

n = number of animals.

- Each bar represents the mean spike ratio per 5 min  $\pm$   
standard error (S.E.M.).

1. ECoG recording was done for an initial control period of 30 min. The mean spike count per 5 min from either the primary or secondary focus during that period was expressed as a ratio of 1.
2. Pimozide (1 mg/kg) was injected i.p., at 30 min (Fig. 24A). Control rats received an equivalent volume of vehicle, i.p., (Fig. 24B).
3. ECoG recording was continued for 20 min more. Then both groups of animals received CF 25-397 (40 mg/kg) i.p., and recording was continued every second half hour for the following 6 hrs.  
During the post-injection period of each animal, the spike ratio per 5 min was calculated as described before.
4. Comparison of effect between pimozide-treated and controls was done using the Mann-Whitney-U-test

+ P = 0.05.

Fig 24A

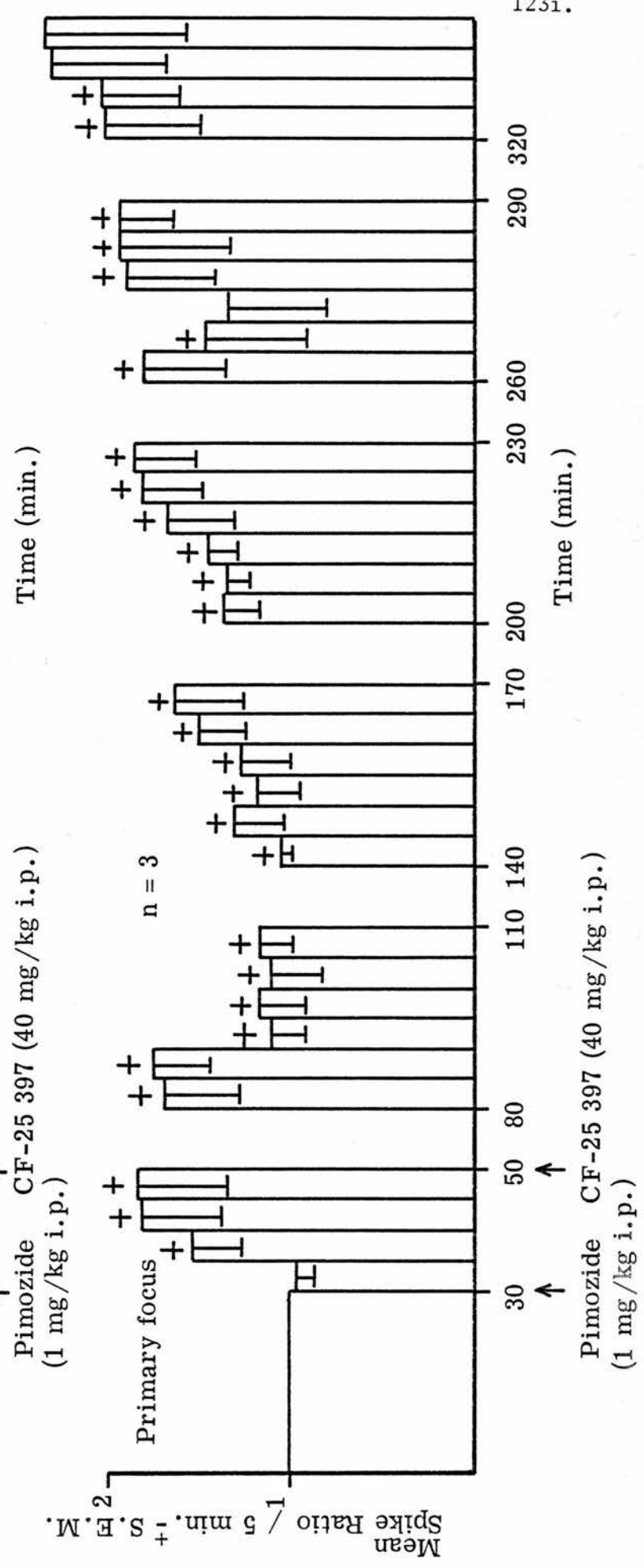
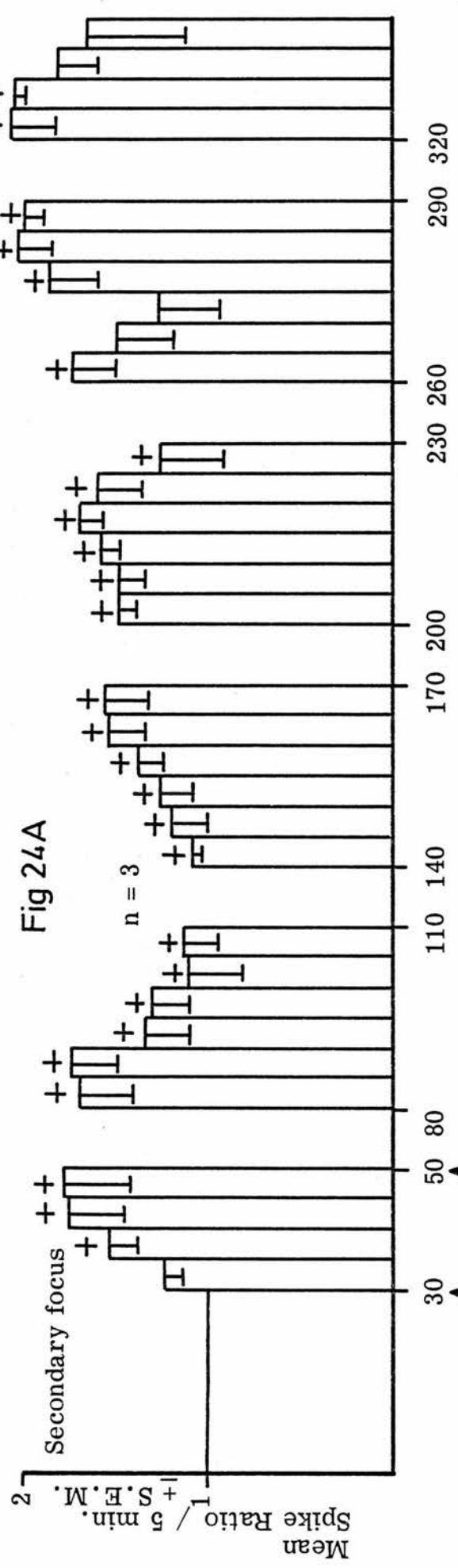
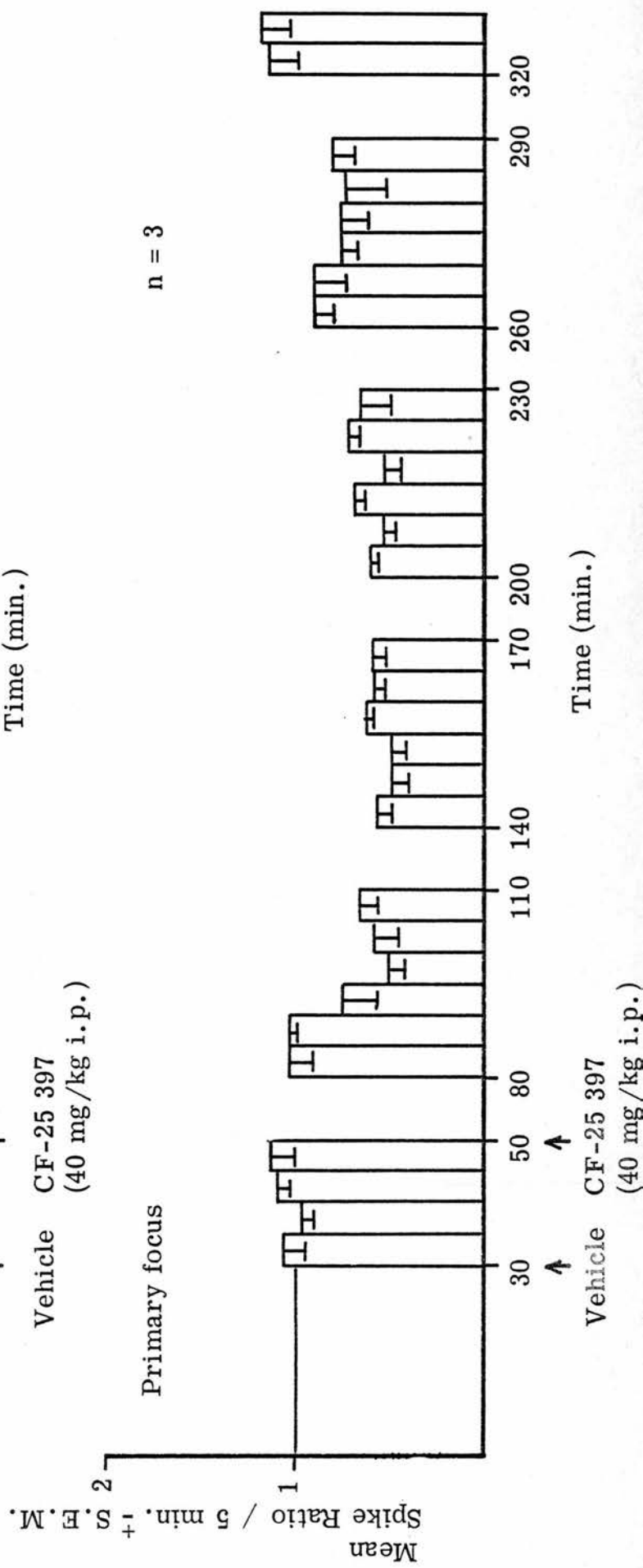
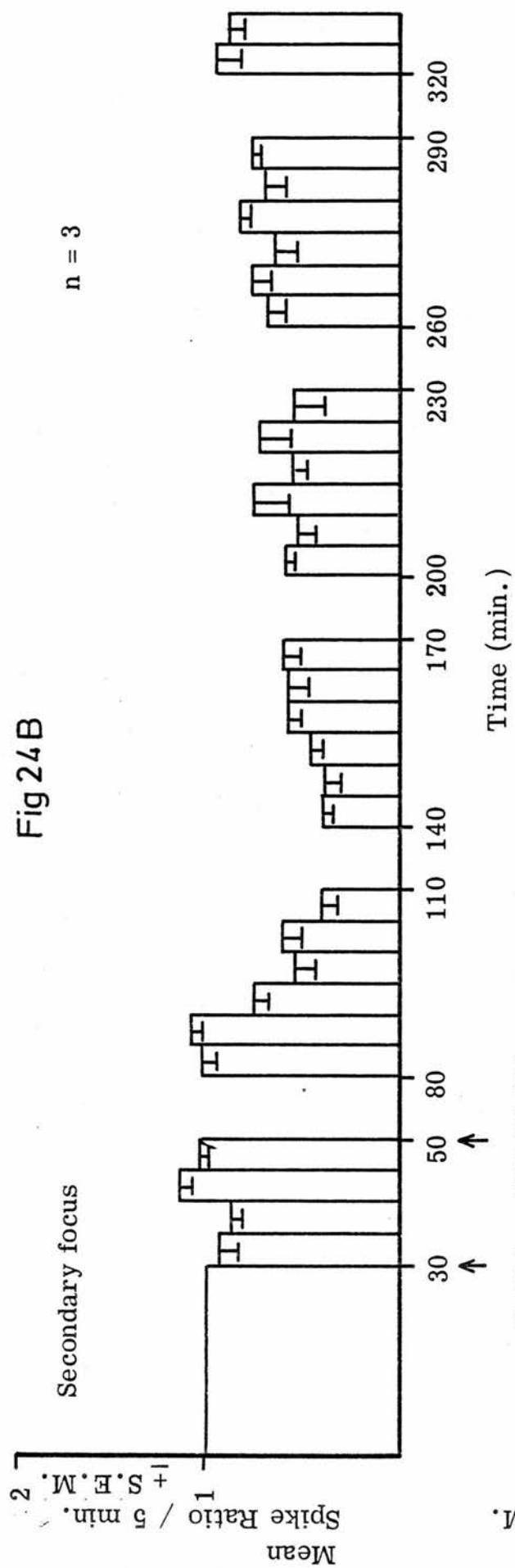


Fig 24B



## 2. Effect of apomorphine

(Figs. 27a and b). The injection of 60  $\mu$ g apomorphine into each striatum significantly and dramatically suppressed the spike activity of both primary and secondary foci, as well as effecting a reduction in motor epileptic manifestation. These effects were more intense than those induced by dopamine and lasted for about 50 min. Behavioural signs of increased locomotion, arousal, and stereotyped gnawing behaviour accompanied these effects.

## 3. Effect of destruction of catecholamine terminals in the striatum on both sides on the development of epileptic activity.

The motor manifestation of bilateral 6-OHDA lesions of both striata included hypokinesia and difficulty in eating and drinking. However, rats were maintained throughout the experiment with supplementary feeding. Other workers also observed these changes with 6-OHDA (Ungerstedt, 1971). The normal reported levels of dopamine and noradrenaline levels in the striatum are 9200 ng/g tissue weight ( $\pm$  700 SEM) and 266 ng/g tissue weight ( $\pm$  13 SEM), respectively, (Tassin et al, 1975). The concentrations of catecholamines in the striatum of saline-injected rats did not differ significantly from the control values reported above.

The bilateral intrastriatal injection of 6-OHDA (20  $\mu$ g) reduced significantly the concentration of dopamine to 10% and 15% in right and left striata, respectively, in comparison to controls (Table 15). Furthermore, the concentration of noradrenaline was significantly reduced in the right and left striata to 26% and 30% respectively, in comparison to controls (Table 16).

Legend to Fig. 25

EFFECT OF BILATERAL INJECTION OF DOPAMINE INTO EACH STRIATUM  
ON EPILEPTIC ACTIVITY.

n = number of rats.

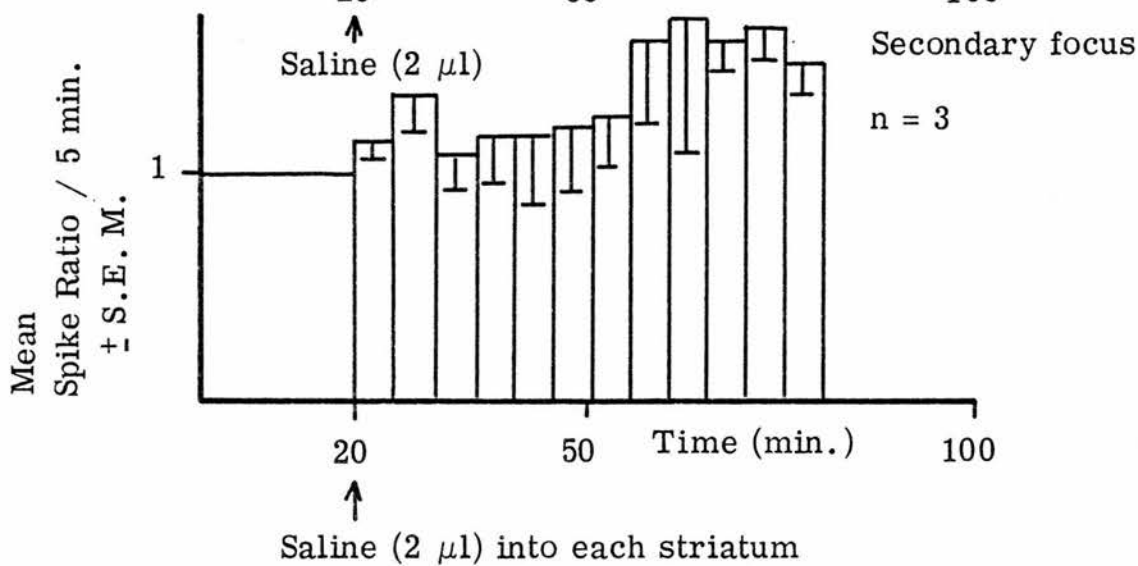
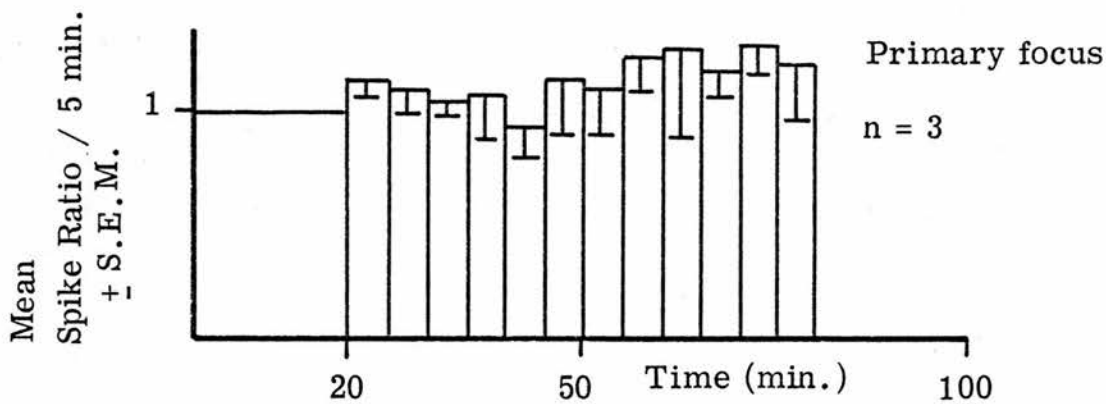
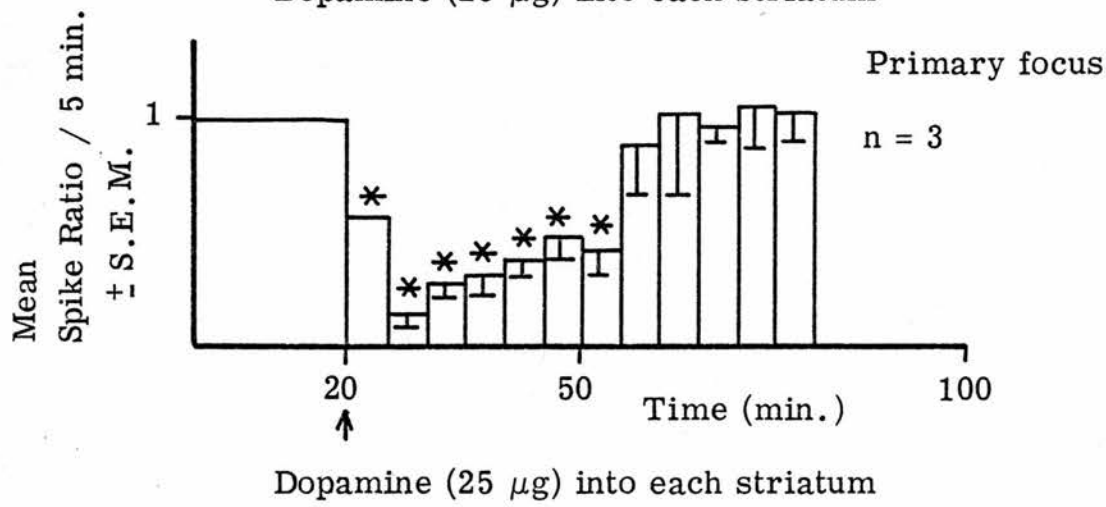
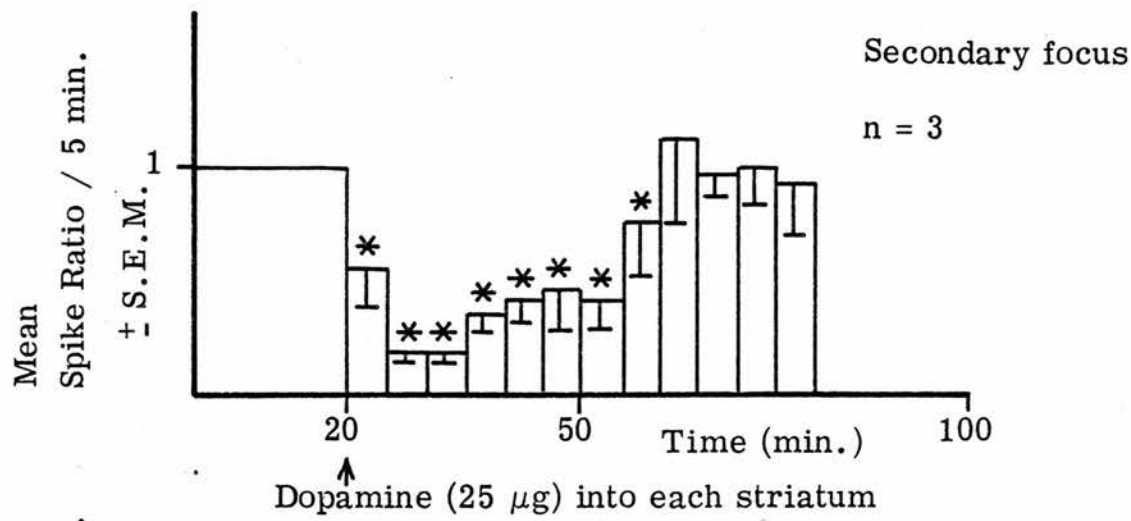
- Each bar represents the mean spike ratio per 5 min  $\pm$  standard error (S.E.M.).

1. ECoG recording was done for an initial control period of 30 min.
2. Dopamine (25  $\mu$ g in 2  $\mu$ l normal saline) was injected into each striatum. Recording was continued for one hour after the injection.

Control animals received 2  $\mu$ l saline into each striatum.

3. Comparison of effect between the dopamine-treated and saline controls was done with the Mann-Whitney-U-test.

\* P = 0.05.





Legend to Fig. 26

EFFECT OF UNILATERAL INJECTION OF DOPAMINE INTO THE RIGHT STRIATUM ON EPILEPTIC ACTIVITY.

n = number of animals.

- Each bar represents the mean spike ratio per 5 min  $\pm$  S.E.M. (standard error).

1. ECoG recording was done for an initial control period of 30 min.
2. Dopamine (25  $\mu$ g in 2  $\mu$ l normal saline) was injected into the right striatum. Controls received 2  $\mu$ l saline into the right striatum. Recording was continued for one hour after injection.
3. Comparison of effect between the dopamine-treated and saline controls was done with the Mann-Whitney-U-test.

\* P = 0.05.

Fig 26 A

124b.

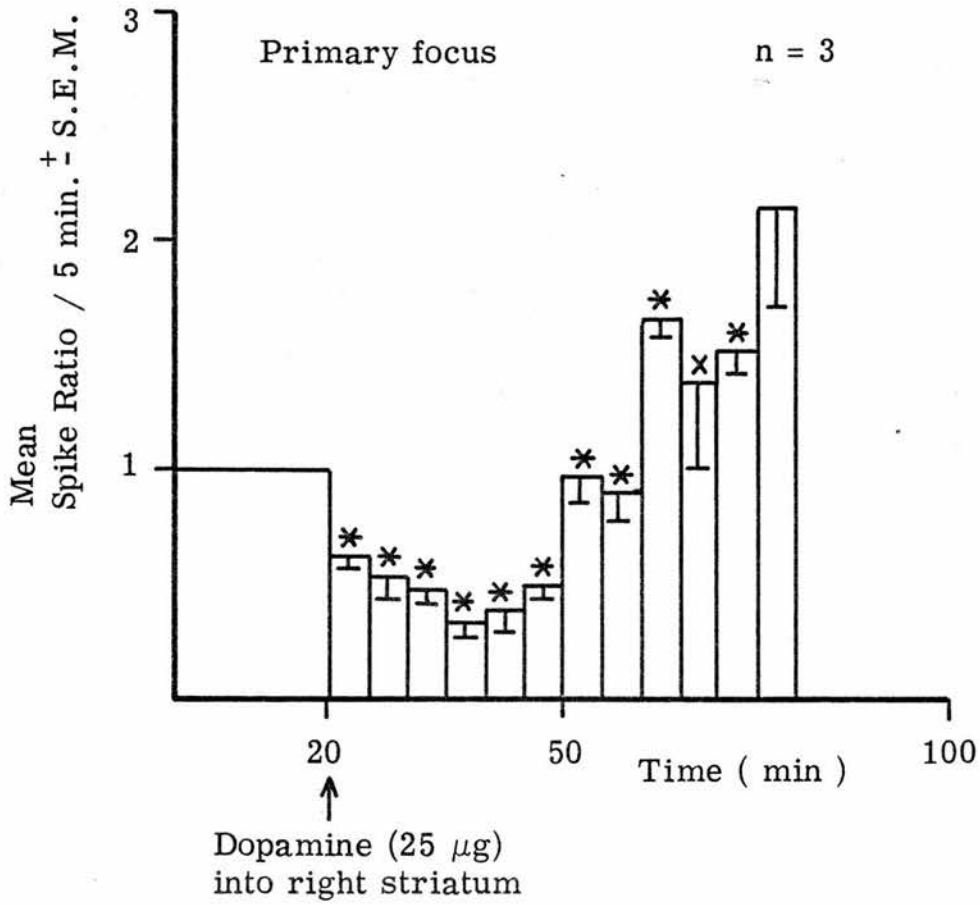
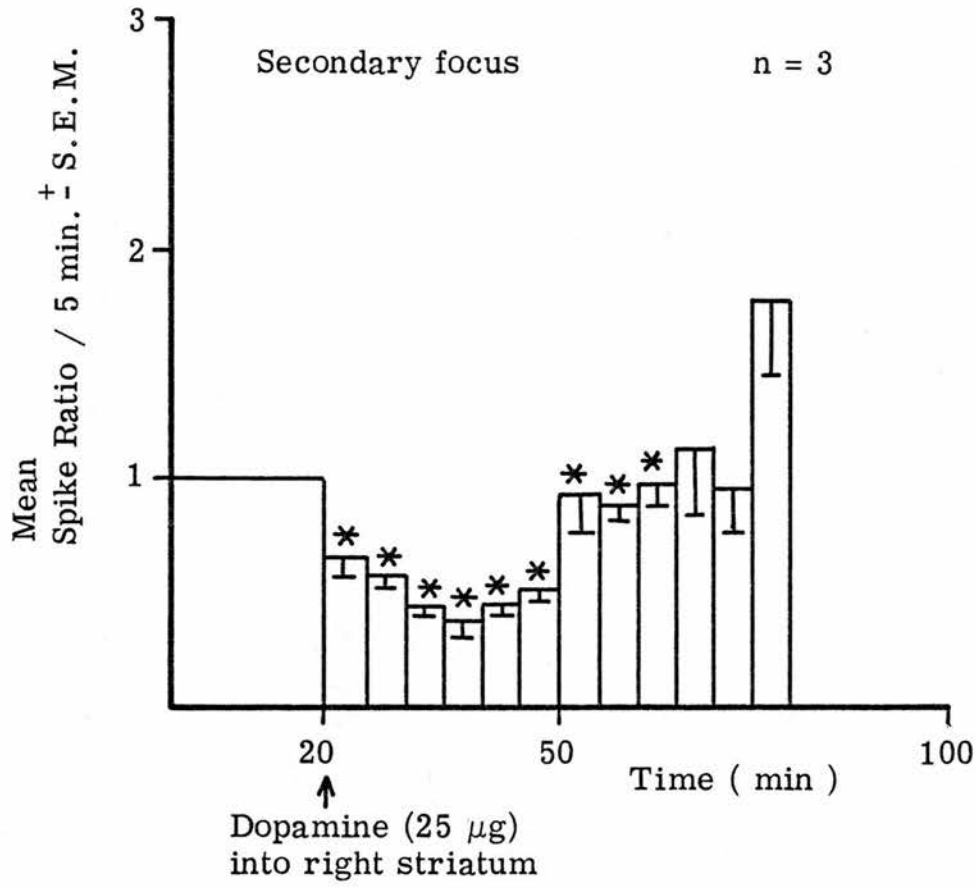
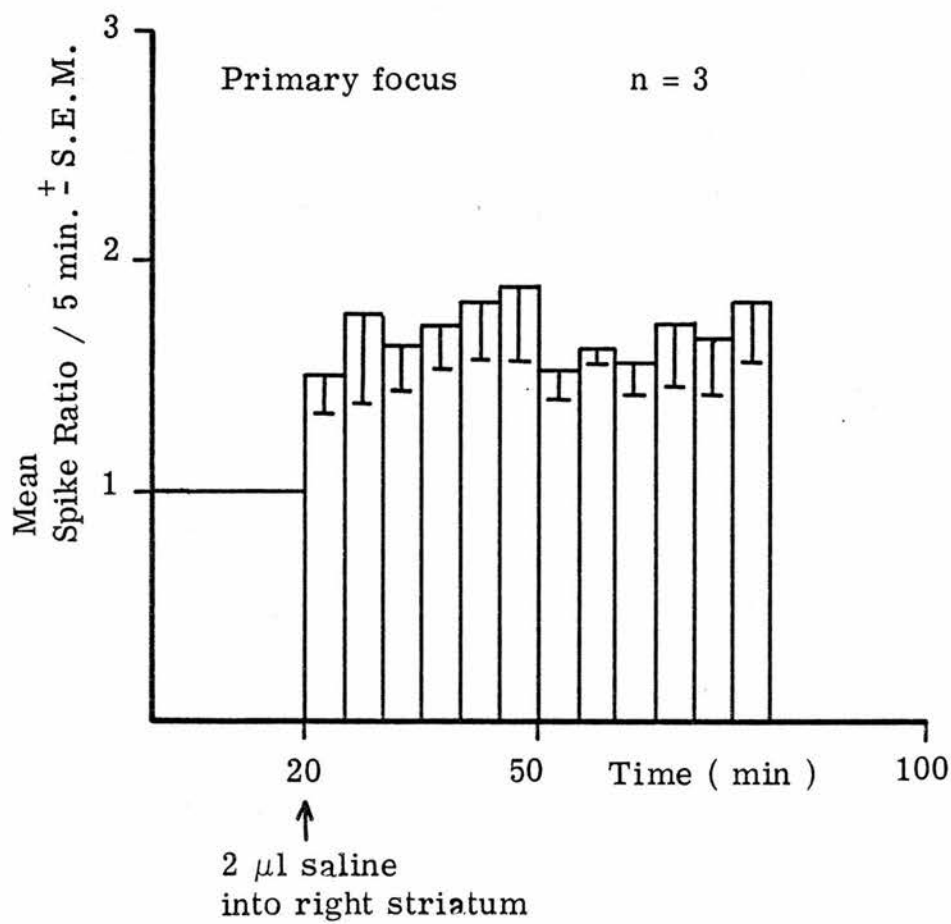
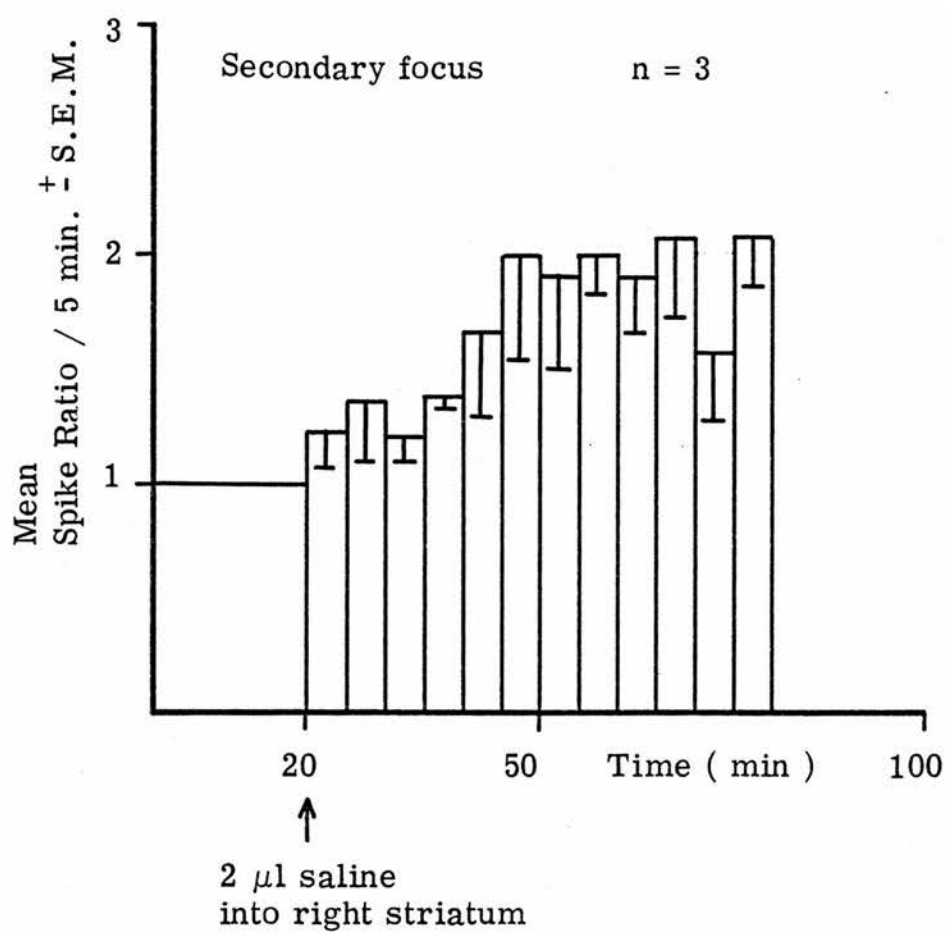


Fig 26B

124c.



Legend to Fig. 27

EFFECT OF BILATERAL INJECTION OF APOMORPHINE INTO EACH STRIATUM ON EPILEPTIC ACTIVITY.

n = number of rats

- Each bar represents the mean spike ratio per 5 min  
+ standard error (S.E.M.).

1. ECoG recording was done for an initial control period of 20 min.

2. Apomorphine HCl (60  $\mu$ g in 2  $\mu$ l/normal saline), was injected into each striatum.

Control rats received 2  $\mu$ l normal saline into each striatum. ECoG recording continued for one hour after the injection.

3. Comparison of effect between drug-treated and control rats was done with the Mann-Whitney-U-test.

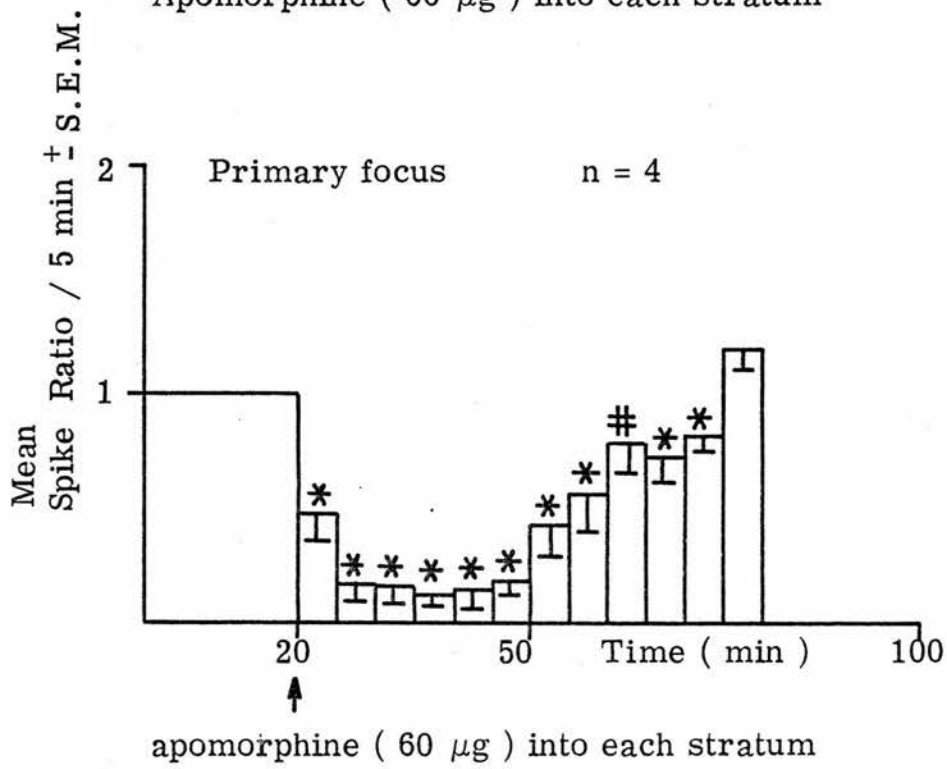
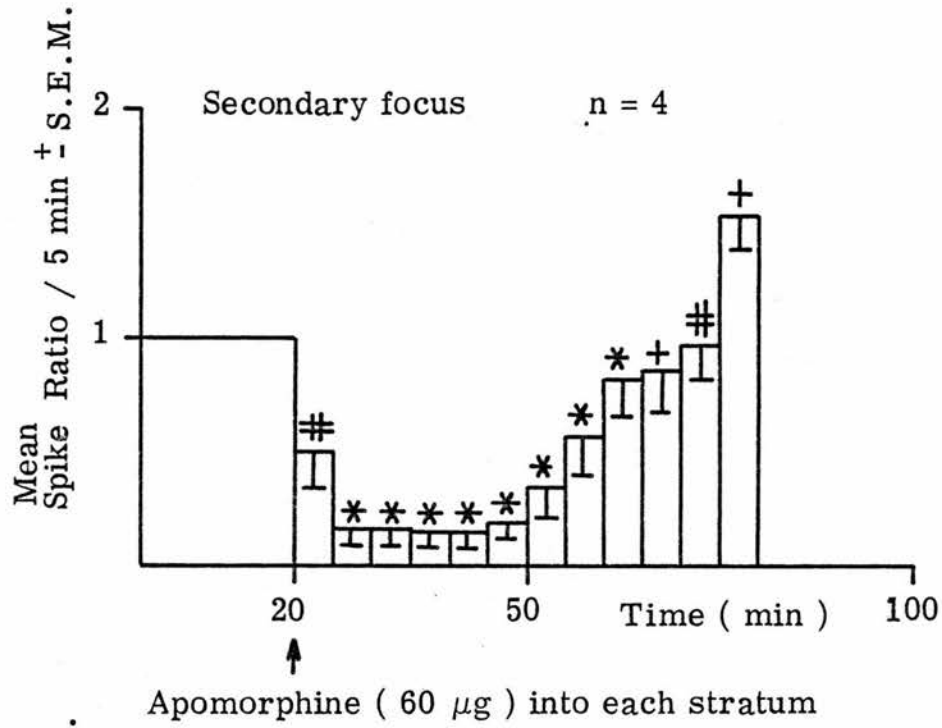
\* P = 0.14

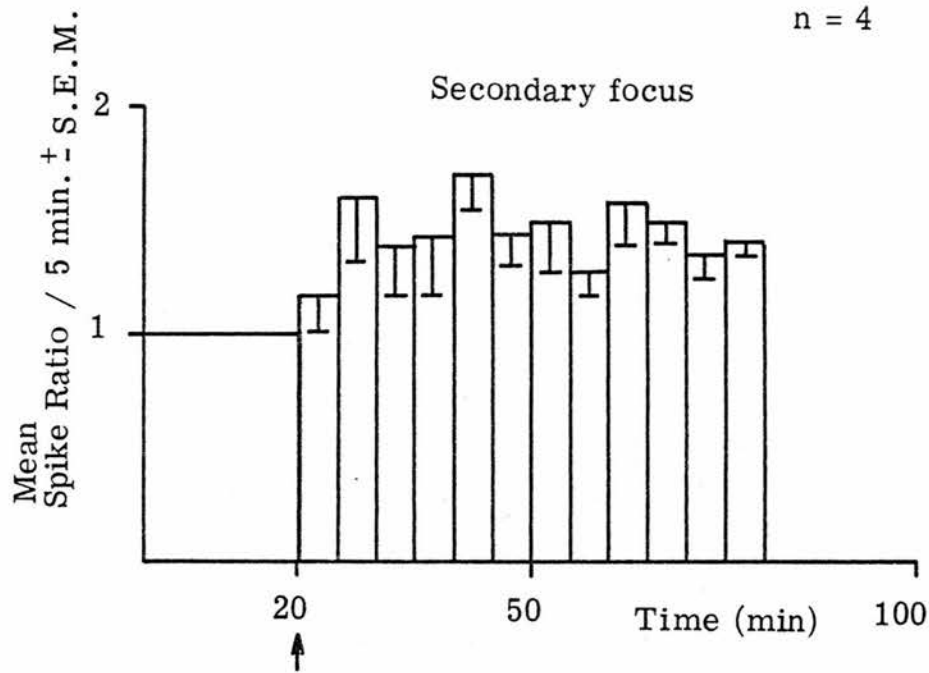
+ P = 0.029

~~##~~ P = 0.05.

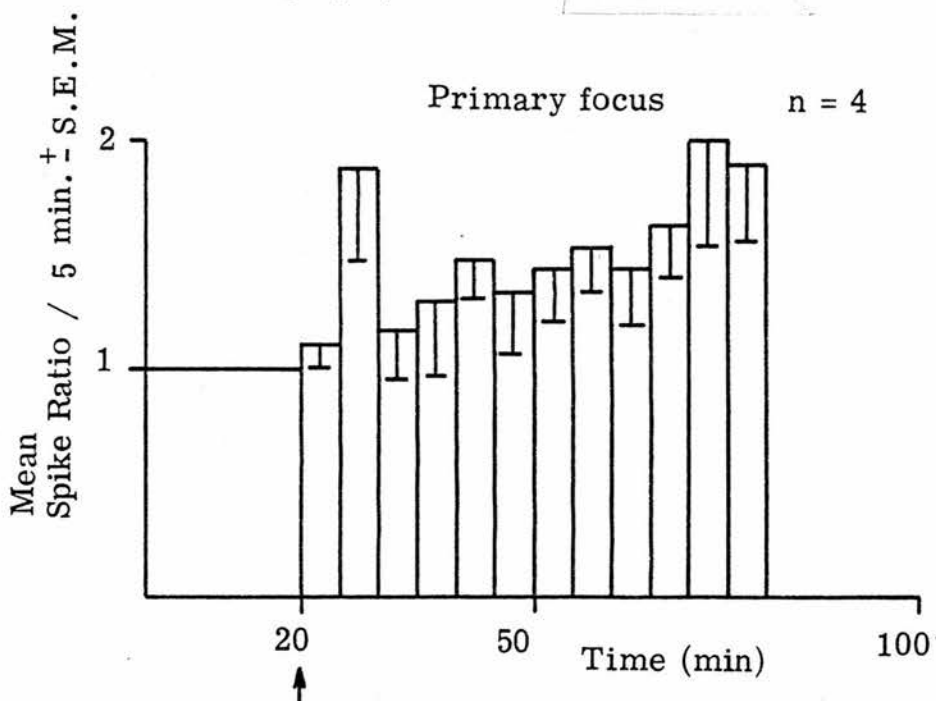
Fig27 A

124d.





Saline ( 2  $\mu$ l ) into each striatum



Saline ( 2  $\mu$ l ) into each stratum

The 6-OHDA lesion in striatum on both sides significantly potentiated the spike frequency of both primary and secondary foci induced by cobalt implantation in comparison to controls (Kruskal-Wallis one-way analysis of variance, Figs. 28a and b). This increase in spike activity was evident within 24 - 48 hrs of cobalt implantation and persisted throughout the period of observation and was associated with more frequent forelimb and whisker twitches.

TABLE 15

EFFECT OF BILATERAL 6-HYDROXYDOPAMINE INJECTION (20  $\mu$ g IN 2  $\mu$ l SALINE)  
ON THE DOPAMINE CONTENT IN EACH STRIATUM OF COBALT-IMPLANTED RATS

- concentrations are expressed as ng/g wet tissue weight

Rat No.	Controls (2 $\mu$ l saline)		6-OHDA (20 $\mu$ g)	
	Right striatum (ng/g)	Left striatum (ng/g)	Right striatum (ng/g)	Left striatum (ng/g)
R1	8679	8762	355	1473
R2	10421	8278	108	132
R3	10913	11679	1501	4180
R4	8360	7997	1033	830
R5	10205	10382	1767	1656
R6	10890	-	-	405
Mean	9911	9420	953 *	1446 *
+ S.E.	416	625	285	445

\*P < 0.001 when compared with the pooled concentration in controls (Student's-t-test, two-tailed, with Bessel's correction)

(-) = sample lost during the assay



TABLE 16

EFFECT OF BILATERAL 6-HYDROXYDOPAMINE INJECTION (20  $\mu$ g IN 2  $\mu$ l SALINE) ON THE NORADRENALINE CONTENT OF EACH STRIATUM IN COBALT-IMPLANTED RATS

- concentrations are expressed as ng/g wet tissue weight

Rat No.	Controls (2 $\mu$ l saline)		6-OHDA (20 $\mu$ g)	
	Right striatum (ng/g)	Left striatum (ng/g)	Right striatum (ng/g)	Left striatum (ng/g)
R1	407	405	38	219
R2	335	425	54	40
R3	402	407	39	162
R4	144	201	94	72
R5	297	206	174	41
R6	219	-	61	48
Mean	301	329	77 *	97 *
+ S.E.	38.7	45.8	19.3	28

\*  $P < 0.001$  when compared with the pooled concentration in controls (Student's-t-test, two-tailed, with Bessel's correction)

(-) = sample lost during the assay

Legend to Figs. 28A and B.

EFFECT OF BILATERAL 6-OHDA LESION OF STRIATUM ON THE DEVELOPMENT  
OF EPILEPTIC FOCI IN COBALT-IMPLANTED RATS.

n = number of animals.

- Each bar represents the mean spike count per min  $\pm$  standard error (S.E.M.).

1. 6-OHDA (20  $\mu$ g in 2  $\mu$ l saline) was injected into each striatum at the time of cobalt implantation in one group of rats. Control rats received 2  $\mu$ l saline into each striatum.
2. Daily 10 min ECoG recording was done for the first 8 days, and then on alternate days until the 14th day.
3. Comparison of effect between drug-treated and saline-treated rats was done with Kruskal-Wallis analysis of variance.

\* P = 0.001 - 0.01

+ P = 0.01 - 0.02

++ P = 0.02 - 0.05.

Fig 28A

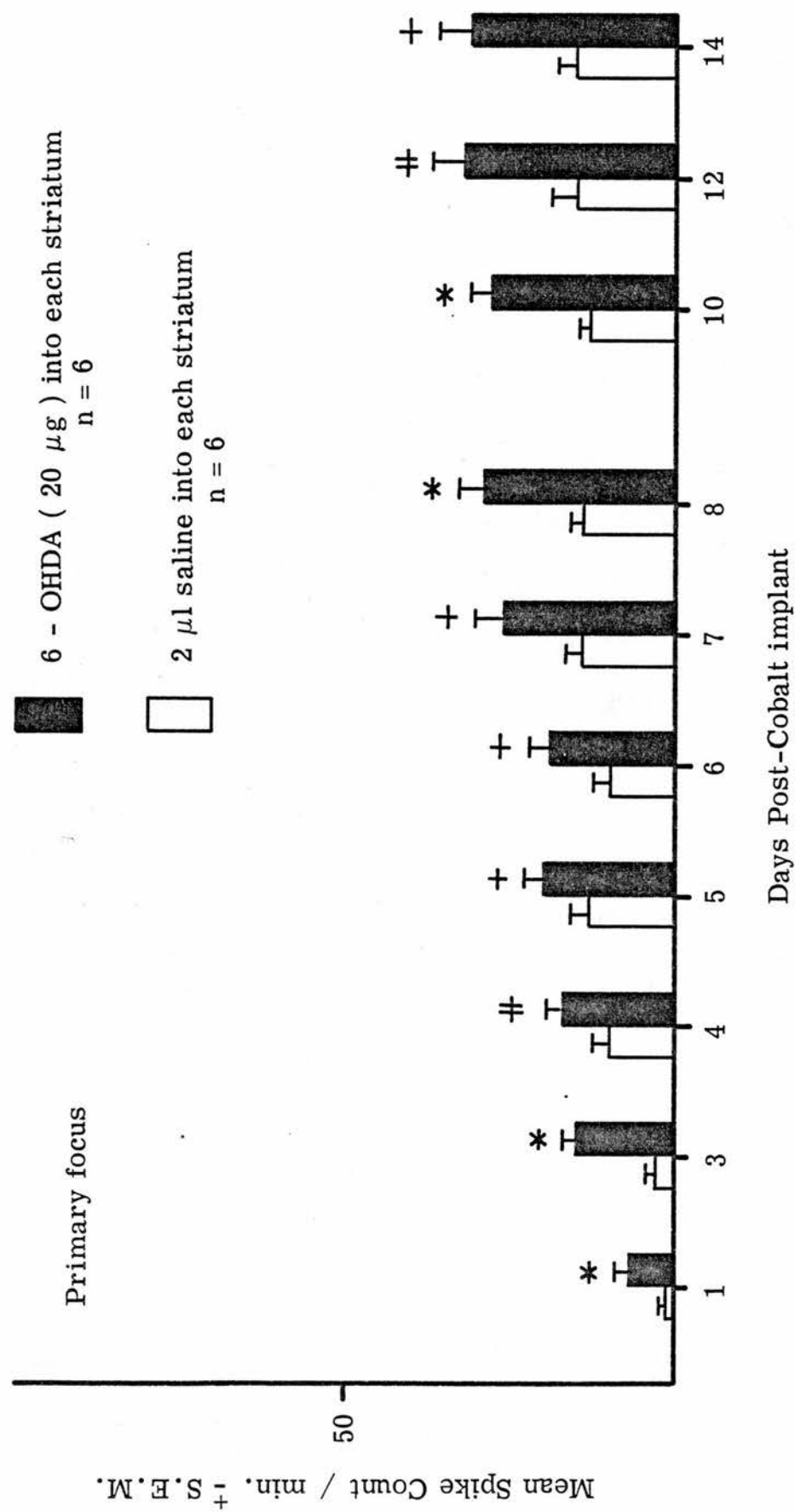
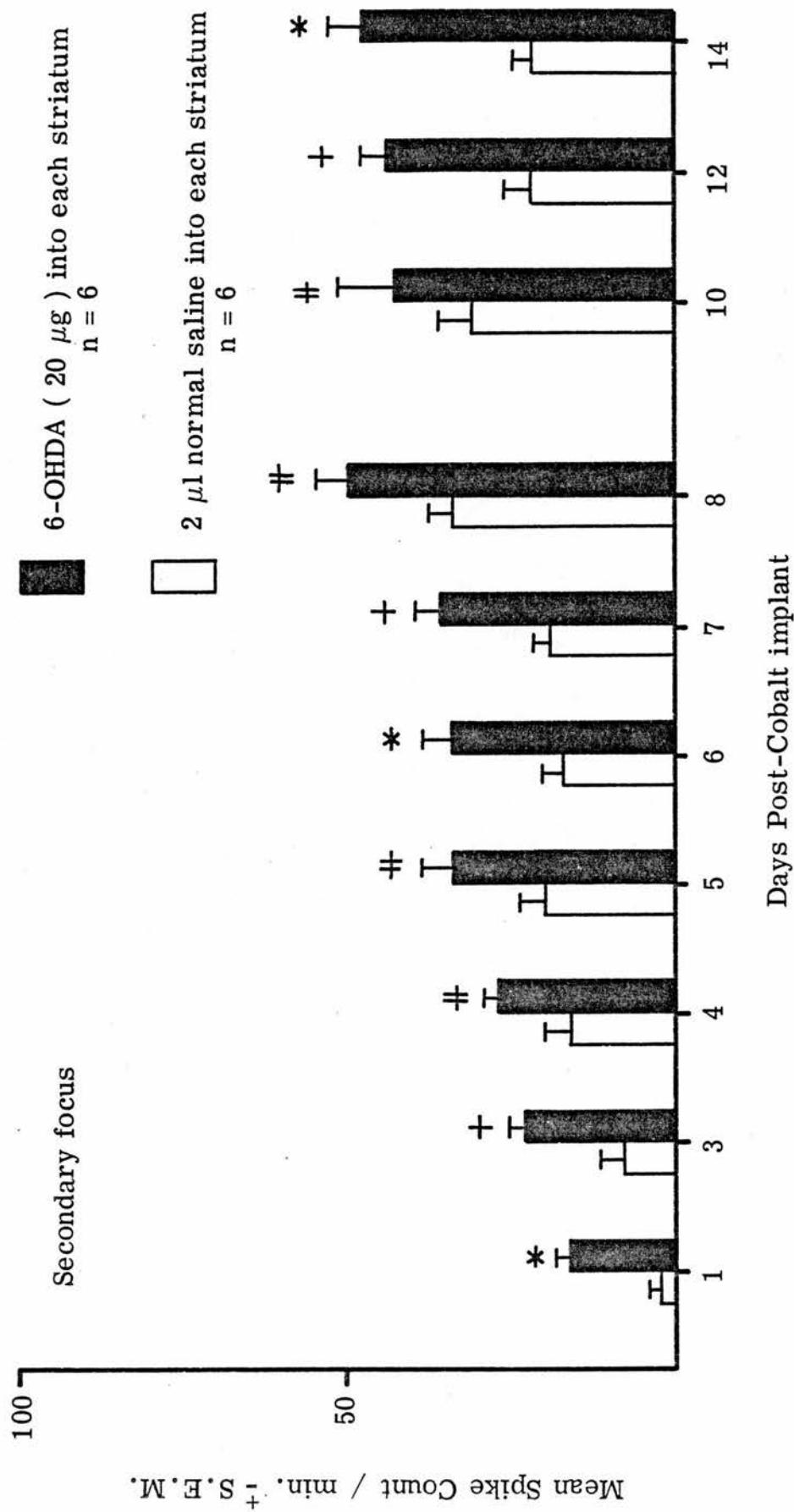


Fig 28B



## DISCUSSION

### Effect of dopamine agonists and antagonists.

In the present work, the time factor in the development of epileptic activity matched the previous data in the cobalt model (Emson & Joseph, 1975, Dow et al, 1972), and added more support to the uniformity of the of the epileptogenic time in cobalt-implanted rats.

All the dopamine agonists used in the present experiments, suppressed the epileptic activity of both the primary and secondary cortical foci, an effect reduced by pimozide. Apomorphine is well known to stimulate the dopamine receptors (Ernst, 1967, Anden, Fuxe and others, 1967). Apomorphine is a quick-acting dopamine agonist. Its anti-epileptic activity which lasted about 40 - 50 min with doses used, tended to be followed by exacerbation of epileptic activity that reached significance level with a dose of 2 mg/kg ( $P = 0.05$ ). Lisuride is also a rapidly acting dopamine agonist (Horowski, 1975, Kehr et al, 1977). However, its effect on epileptic activity lasted longer than that of apomorphine, probably due to the more rapid metabolism of the latter.

Bromocryptine is known to stimulate the dopamine receptors directly (Corrodi et al, 1973), Johnson et al, 1976). It differs from apomorphine by the delayed onset of its effects, and that its actions are blocked by presynaptic depletion of catecholamines whether induced by reserpine or alpha-methyl tyrosine, which makes bromocryptine behave like a partial dopamine agonist (Corrodi et al, 1973, Johnson et al, 1976). Moreover, bromocryptine inhibits dopamine re-uptake (Silbergeld & Pfeiffer, 1977). It is known that low doses of bromocryptine (less than 1 mg/kg) inhibited

motor function and this effect was attributed to stimulation of dopamine auto-receptors (Snider et al, 1976). Similar effect was described with low doses of apomorphine (less than 0.5 mg/kg) (Strombom, 1976). In contrast to apomorphine, the suppressant effect of 10 and 20 mg/kg bromocryptine on epileptic activity occurred within 3 and 1 hrs, respectively, after intraperitoneal injection. The fact that the latent period was reduced with the larger dose fits with the previous reports (Snider et al, 1976). In contrast to apomorphine, the anti-epileptic effect of bromocryptine lasted more than 6 hrs and the animal had recovered from its effect after 24 hrs.

The lag phase before the onset of action of bromocryptine might represent the time necessary for the formation of an active metabolite. It might be that intact presynaptic events are involved in the formation of such a pharmacologically active moiety. The similarity between the structure of the bromocryptine molecule and that of L-tyrosine makes it possible for bromocryptine or one of its peripheral metabolites to be acted upon by tyrosine hydroxylase to produce an active substance. Furthermore, presynaptic uptake of bromocryptine might occur by utilization of tyrosine uptake mechanisms. In this respect, the intraneuronal accumulation of the related ergot alkaloid dihydro-ergotoxin was demonstrated (Meier-Ruge & Iwaghoff, 1976). Moreover, the hypothermia resulting from bromocryptine in rats, itself a phenomenon thought to be mediated by dopamine receptor stimulation, was decreased by pretreatment with SKF 525A, a potent inhibitor of hepatic microsomal enzymes. This suggests that bromocryptine depends, at least in part, on hepatic metabolism for its conversion into active compounds that possess hypothermic and dopaminergic activity. However, there is no

evidence yet of such active metabolite of bromocryptine, and its isolation from the urine of rat or man might be of therapeutic value in treatment of Parkinsonism, endocrine disorders, such as acromegally and galactorrhea and possibly focal epilepsy. Bromocryptine, however, does cross the blood-brain barrier and it is known to interact with the acute effects of apomorphine and amphetamine (Dray & Oakley, 1976). Furthermore, it has been shown to have a direct effect in inhibiting prolactin-secreting cells of the hypophysis (Pasteels et al, 1971).

The chronic daily administration of bromocryptine attenuated the rate of development of epileptic activity, particularly in the secondary foci. This effect suggests that persistent stimulation of dopaminergic mechanisms might be a useful adjunct in the management and prophylaxis of focal epilepsy.

CF 25-397 is a dopamine agonist which is thought to be more selective in stimulating the nigro-striatal system by the fact that it does not induce stereotyped behaviour, even in large doses (Jaton et al, 1976).

In the present work, this drug was found less effective than bromocryptine in suppressing epileptic activity. At a dose of 40 mg/kg given i.p., CF 25 reduced the spike frequency of both primary and secondary foci, while at 20 mg/kg it had no significant effect. Even at the effective dose, CF 25 did not induce stereotypy thus confirming its reported selectivity as dopamine agonist. However, the latent period of its action was shorter than that seen with bromocryptine. Moreover, its anti-epileptic effect seemed to wear off earlier than bromocryptine. The formation of active metabolites was also suggested,

in part, as a possible mechanism for its action. However, it has been shown that inhibition of microsomal hepatic enzymes by SKF 525 reduced, but did not block, the hypothermia induced by CF 25 in rats (Silbergeld & Pfeiffer, 1977).

Pretreatment with the selective dopamine receptor blocker, pimozide, potentiated the epileptic activity of cortical foci in cobalt-implanted rats. Moreover, it blocked the anti-epileptic activity induced by all the dopamine agonists mentioned above.

Thus, it seems that dopamine receptor stimulation inhibited epileptic activity while blockade of those receptors had the opposite effect. The finding that the anti-epileptic effects of the different dopamine agonists was dose-dependent adds more support to this point. These results also confirm the work of Ashcroft et al (1974), who showed that dopamine agonists suppressed cortical epileptic activity while dopamine antagonists potentiated it in cobalt-implanted rats, and conversely adrenergic stimulants increased while adrenergic blockers inhibited epileptic activity in the same model. Therefore, there seemed to be a sort of balance in catecholamine neuro-transmitters, with dopamine tending to inhibit and noradrenaline to increase the excitability of cortical neurosis.

There is evidence that focal motor epilepsy, the two types of reflex epilepsies (including audiogenic seizure in mice and photogenic seizure in the baboon), and the later stages of electro-convulsive shock are all alike in that proprioceptive afferents, mediating impulses arising from motor activity, play an important role in the evolution of the seizure response (Chauvel et al, 1975, Naquet et al, 1975, Reguin &



Pillerd, 1966). Thus, dopamine, apomorphine and other dopamine agonists may act directly or indirectly on this recurrent system in inducing their anti-convulsive effects. In the present work and that of Ashcroft et al (1974), the anti-convulsive effect of dopamine agonist and antagonists is well demonstrated in the cobalt model of focal epilepsy. In the reflex epilepsies, it has been shown that audiogenic seizures are enhanced by pretreatment with reserpine and this enhancement can be reversed by giving L-DOPA (Boggan & Seiden, 1971). Furthermore, apomorphine and ergocornine prevent or delay audioshock in the audio-sensitive strain (DBA/2) of mice (Anlezark & Meldrum, 1972). L-DOPA, apomorphine, and piribedil inhibited completely the myoclonic response to intermittent photic stimulation in the photo-sensitive baboon while haloperidol potentiated it (Meldrum et al, 1972, 1975a). Thus, it seems that epileptic discharges from cortical foci induce motor epileptic phenomenon and the resulting motor activity perpetuate, at least in part, these cortical discharges via proprioceptive afferents. This mechanism tends to involve dopaminergic control at one stage, hence the suppressant effect of dopamine agonists and the potentiating effect of antagonists in these forms of sensory epilepsy.

However, drugs which act as dopamine agonists don't always show anti-epileptic activity and some species and model variations do exist. Thus, apomorphine protects against electroshock in rats, but not in mice (McKenzie & Soroko, 1972), and it may potentiate chemically-induced seizures by allylglycine in the baboon (Meldrum et al, 1975b), or pentylenetetrazol-induced seizure in rodents (Soroko & McKenzie, 1970). Bromocryptine, however, seemed to be an effective anti-

convulsant in acute and chronic experiments in cobalt-implanted rats in the present work; besides, no species or model variation are reported for this drug yet in experimental epilepsy. Thus the present findings might justify a limited clinical trial for bromocryptine in focal epilepsy.

The possibility still exists that changes in the epileptic phenomena observed in the present work in cobalt-implanted rats as well as those reported in other forms of 'sensory epilepsy' might be due to drug interaction other than those on the dopamine system. Apomorphine and haloperidol inhibit the uptake of gamma-aminobutyric acid by brain slices (Harris et al, 1973). Bromocryptine and apomorphine also stimulate 5-HT receptors. The action of dopamine drugs on noradren-ergic system cannot be excluded. Nevertheless, the effect of dopamine agonists and antagonists can most readily be explained in terms of activation and inhibition of the dopamine receptors respectively, leading to cortical desynchronization and hypersynchronization, respectively.

#### Dopamine agonist and antagonists and dopamine receptor binding

Considerable evidence support the view that the turnover of brain dopamine is regulated in part by postsynaptic dopamine receptors. Thus, dopamine receptor antagonists have been found to stimulate, and agonists to inhibit dopamine turnover (Carlsson & Lindquist, 1963, Anden et al, 1967, Nyback & Sedval, 1970). Dopamine is known to be a highly specific stimulant of adenyl cyclase activity in the striatum as well as other central areas (Kebabian & Greengard, 1971, Horn et al, 1974). Consequently, adenyl cyclase activation has been considered to be

representative of dopamine receptor response (Kebabian et al, 1972).

[<sup>3</sup>H] dopamine and [<sup>3</sup>H]haloperidol bind with high affinity and selectivity to the post-synaptic dopamine receptors in membrane preparations of the calf caudate nucleus (Creese et al, 1975). The binding of these ligands to the dopamine receptor differs from the data obtained with dopamine-sensitive adenylyl cyclase (Kebabian et al, 1972, Iversen et al, 1975). Butyrophenones represent the clinically most effective class of neuroleptics as reflected in their high affinity for [<sup>3</sup>H] haloperidol binding sites. However, they are weak inhibitors of adenylyl cyclase (Burt et al, 1975). These discrepancies indicate that the recognition site of the dopamine receptor is not identical with dopamine-sensitive adenylyl cyclase.

The binding of both ligands shows marked regional variation with greatest density in the caudate, putamen, globus pallidus, nucleus, accumbens and olfactory tubercle, areas that are rich in dopaminergic terminals (Creese et al, 1975). It has been shown that 6-OHDA lesions of the nigro-striatal pathway does not alter the binding of these ligands to the dopamine receptor, thus pointing to a post-synaptic location of these binding sites. However, more recently, Schwartz et al, (1978) demonstrated that at least 35% of [<sup>3</sup>H]haloperidol binding in the striatum is due to binding to presynaptic terminals from fibers originating from the cerebral cortex, and it was suggested that this binding might mediate at least in part, the action of anti-psychotic drugs. However, [<sup>3</sup>H] dopamine binding has not been reported for any presynaptic site yet. The binding of dopamine agonists and antagonists to these ligands indicates their selectivity in associating with the dopamine receptor

TABLE 17A

SELECTIVITY OF BINDING OF DOPAMINE AGONISTS AND ANTAGONISTS  
TO THE DOPAMINE RECEPTORS IN CALF BRAIN MEMBRANES

- (From Burt, Creese and Snyder, 1976)

Inhibition of [ $^3\text{H}$ ] haloperidol and [ $^3\text{H}$ ] dopamine by drugs

Drug	Ki	
	[ $^3\text{H}$ ] Haloperidol	[ $^3\text{H}$ ] Dopamine
	(nM)	(nM)
Dopamine	670	17.5
Apomorphine	51	8.6
Bromocryptine	2.5	106
Pimozide	0.81	5300
Haloperidol	1.4	920
(+)-Butaclamol	0.54	80
(-)-Butaclamol	700	> 10.000

The rate constant of association (Ki) of each drug to either ligand was calculated by the formula:-

$$K_i = \frac{IC_{50}}{1 + C/K_D}$$

IC<sub>50</sub> = The concentration of drug required to inhibit specific binding by 50%.

C = The concentration of radioactive ligand (1 to 2 nM for [ $^3\text{H}$ ] haloperidol and 5 nM for [ $^3\text{H}$ ] dopamine).

K<sub>D</sub> = Dissociation constant of the labelled ligand (2 nM for [ $^3\text{H}$ ] haloperidol and 20 nM for [ $^3\text{H}$ ] dopamine)

(See Table 17A). Drugs lacking dopaminergic properties display negligible binding. The relative potency of various agonists and antagonists on the binding of the two ligands parallels their pharmacological actions at dopamine receptor sites (Burt et al, 1976). Dopamine agonists have 6-38 times more affinity for [ $^3\text{H}$ ] dopamine than [ $^3\text{H}$ ] haloperidol binding sites. By contrast, dopamine antagonists have 20-12,000 times more affinity for [ $^3\text{H}$ ] haloperidol than [ $^3\text{H}$ ] dopamine binding sites. Ergot derivatives have a substantial affinity for both types of binding (Burt et al, 1976). This difference in substrate specificity suggests that dopamine and haloperidol label different portions or states of the dopamine receptor (Creese et al, 1975). There might be distinct dopamine receptor sites, binding agonists and antagonists, respectively. Alternatively, one dopamine receptor might inter-convert between conformations which have high affinities for either agonists or antagonists and this might explain the pharmacological properties of dopamine agonists and antagonists in terms of their affinity for the two-state model of the receptor (Snyder, 1975). According to this model, pure agonists have greater affinity for [ $^3\text{H}$ ] dopamine than [ $^3\text{H}$ ] haloperidol binding sites while the reverse is true for antagonists. Mixed agonists-antagonists should have similar affinities for both binding sites. Table 17B shows the potency of different agonists and antagonists used in the present work in inhibiting [ $^3\text{H}$ ] dopamine and [ $^3\text{H}$ ] haloperidol binding.

Thus, in reducing [ $^3\text{H}$ ]dopamine binding, apomorphine is the most potent. It has been shown that the number of apomorphine binding sites (Seeman et al, 1976), is three times as high as dopamine binding sites. In the present experiments, apomorphine was found to induce a more

TABLE 17B

POTENCY OF SOME DOPAMINE AGONISTS AND ANTAGONISTS IN THEIR BINDING TO THE DOPAMINE RECEPTOR

- (After Creese, Burt and Synder, 1975)

Drug	IC <sub>50</sub>	
	[ <sup>3</sup> H] Haloperidol	[ <sup>3</sup> H] Dopamine
	<u>(nM)</u>	<u>(nM)</u>
Dopamine	1000	22
Apomorphine	150	6
Haloperidol	2.5	500
Pimozide	1.3	2500
(+)-Butaclamol	1.3	130
(-)-Butaclamol	1.300	> 10.000

IC<sub>50</sub> values are the concentrations (nM) of each drug required to inhibit the specific binding of either 5 nM [<sup>3</sup>H] dopamine or 2 nM of [<sup>3</sup>H] haloperidol by 50%.

intense inhibition of epileptic activity than dopamine when injected into the striatum of cobalt-implanted rats.

Bromocryptine is of lower potency than apomorphine in reducing [ $^3\text{H}$ ] dopamine binding. Apomorphine also reduces [ $^3\text{H}$ ] haloperidol binding, but is only 4% as active in this respect than for dopamine binding. However, bromocryptine is more potent than apomorphine in reducing [ $^3\text{H}$ ] haloperidol binding. These data indicate that both apomorphine and bromocryptine behave as mixed agonist-antagonist at dopamine receptor in brain membrane (Burt et al, 1976). Moreover, apomorphine (Iversen et al, 1975) and bromocryptine (Pagnini et al, 1978), behave as agonist-antagonists on adenylyl cyclase. No data are available for lisuride and CF 25-397 binding to dopamine receptor. However, like other ergot preparations, they might have mixed agonist-antagonist properties. In the present work, apomorphine was much more potent than bromocryptine in suppressing epileptic activity of cortical foci. The suppression of epileptic activity produced by 2 mg/kg (5.6  $\mu\text{mol/kg}$ ) apomorphine was approximately equivalent to that caused by 20 mg/kg bromocryptine (26.6  $\mu\text{mol/kg}$ ).

Lisuride, however, seemed more potent than apomorphine in its anti-epileptic effect. At a dose of 0.5 mg/kg, lisuride dramatically suppressed epileptic activity while apomorphine induced only a mild inhibition at this dose. Lisuride, at a dose of 1 mg/kg suppressed all epileptic activity, while apomorphine at a dose of 2 mg/kg severely reduced it and larger doses were used by Ashcroft et al (1974) to obtain a complete suppression of epileptic activity in cortical foci.

It has been shown that systemically administered apomorphine



accumulates well in dopamine receptor-rich areas, but poorly in the cerebellum. This may indicate that most of regional apomorphine accumulation is due to dopamine receptor binding (Melzacka et al, 1978). This may also apply to other dopamine agonists such as bromocryptine or their active metabolites.

It has been shown that apomorphine-induced gnawing depends on striatal concentration of apomorphine (Melzacka et al, 1978). It is possible that apomorphine elimination, and possibly that of other dopamine agonists, follows that for a two-compartment open model. The compartment in which apomorphine has a long half-life probably represents the drug bound to dopamine receptor, while the compartment from which the drug is rapidly eliminated represents apomorphine that is not bound or non-specifically bound. This may explain the differences in the duration of action between the short acting apomorphine or lisuride and the longer acting bromocryptine and CF 25. It has been shown that haloperidol prevents the accumulation of apomorphine in the striatum. This contributes for the antagonistic action of haloperidol to apomorphine but at least in part, it may be a result of the competition between the two drugs for the binding sites of the dopamine receptor (Vetulani et al, 1978). Similar mechanisms could contribute to the antagonistic action of pimozide to apomorphine and other dopamine agonists.

#### Behavioural arousal, ARAS, and anti-epileptic activity of dopamine agonists.

It is noticed during all experiments that increased arousal of the animal is associated with reduced spike activity, i.e. cortical desynchronization while sedation is associated with cortical hypersynchrony.



The state of alertness or arousal may be regarded as the resultant state of a number of sub-systems in dynamic equilibrium. These sub-systems could be specified biochemically, anatomically, or functionally, which may overlap, and could be manipulated pharmacologically, surgically or by alteration of physiological input to the brain, respectively.

Selfridge (1948), found that in a conducting network, such as that existing in the brain or the heart, certain types of stimuli could initiate rhythms of electrical activity synchronizing the firing of large groups of units. These rhythms could be damped down by the introduction of new inputs, provided these were not in phase with the standing rhythms, and strengthened by a reduction of input or by further in-phase input. The procedures tending to strengthen standing rhythm could be exemplified by the effects of eye closure on cortical EEG, namely the appearance of alpha-rhythm in occipital areas and the 11-12 Hz u-rhythm of the motor cortical areas involved. However, if cortical input is increased by opening the eyes or by arithmetical mental handling, then these rhythms are disturbed, and the EEG returns to the more varied waking form.

The ascending reticular activating system (ARAS) exerts a de-synchronizing tone on the cortex, thus mediating arousal. Stimulation of the ARAS in cats has been reported to cause increased vigilance with an increased sensitivity to all sorts of stimuli (Sheard & Flynn, 1965), while lesions of this system result in a state of hypersomnia and cortical hyper-synchrony (Lindsley, 1960). Koella & Czioman (1966), have demonstrated that raphe activation causes inhibition of ARAS.

The action of the raphe and ARAS on the cortex appear to be diametrically opposed, and the state of cortical arousal at any one time will be a reflection of, amongst other things, of the balance between both systems. The cortical hypersynchrony developed in the four stages of the non-REM sleep and which are used to characterize each stage, are partly generated by reduction in the activity of ARAS, the direct synchronizing tone of the raphe itself, reduction of cortical input as well as abolition of visual input to the cortex. In the waking state, ARAS in desynchronizing the cortex, increases the data-handling capacity. Thus, a novel stimulus arising to the cortex is first recognised as novel and then the cortex sends information to the ARAS which responds by amplifying this signal and relaying it back to all areas of the cortex resulting in general arousal which can be caused by non-intense stimuli.

Studies employing anterograde degeneration (Nauta & Kuypers, 1958, Lynch et al, 1973) and retrograde transport techniques (Robertson & Travers, 1975) indicate that the reticular formation sends a small number of diffusely arranged fibers through the diencephalon towards the striatum. The cells of origin of these fibers are mainly located at the caudal mesencephalic and pontine levels. This projection is suggested to be part of the diffuse ARAS. More recently, it has been shown that the striatum via the substantia nigra, sends descending fibers to the reticular formation and superior colliculus (See Fig. 29), from the latter fiber connection exist to the reticular formation to constitute the tectoreticular fibers, as well as to the spinal cord, constituting the tectospinal tract. It is by these descending connections, as well as by ascending fiber connection to the cerebral cortex, that the striatum exerts its effect in producing or potentiating arousal, as well

as exerting motor regulatory influence. It is well known that a major site of action of dopamine agonists is on the dopamine receptors in striatum and mesolimbic system. Thus, it is likely that stimulation of the striatal neurons by these drugs might, at least in part, increase the activity of ARAS via the fiber connections, described above, and augment arousal, thus desynchronizing the cortex. Moreover, the increased motor activity resulting from striatal stimulation also increased the data handling with consequent tendency to desynchronization.

#### Possible interactions between dopamine and acetylcholine.

It is well known that acetylcholine is the main transmitter in ARAS. Several diffuse cholinergic pathways are the source of acetylcholine from the cerebral cortex (Pepeu, 1973). In particular, there is one system of fibers that can be stimulated through the mesencephalic reticular formation, another originates from or passes through the septum, and there is also an intracortical system. It has been shown that dopamine agonists stimulate cholinergic neurons, and thus increased acetylcholine output from the cerebral cortex, while dopamine antagonists had the opposite effect (Pepeu & Bartolini, 1968, Mantovani et al, 1977). Pepeu & Bartolini (1968), investigating acetylcholine output from the cerebral cortex of the cat, demonstrated that EEG desynchronization induced by L-DOPA was associated with an increase in acetylcholine output quantitatively similar to that occurring after amphetamine or stimulation of the reticular formation.

This is in contrast to the situation in the striatum where dopamine agonists inhibited while antagonists of dopamine stimulated

cholinergic neurons (Stadler et al, 1973). Disturbance of acetylcholine-dopamine balance in the striatum is known to induce various clinical extrapyramidal syndromes such as Parkinsonism, chorea. Thus, one might be tempted to believe that such a balance does not occur in the cerebral cortex, since activation of both systems tends to be in the same direction, that is, cortical desynchronization. It has been shown that the putative catecholamine releasers, amphetamine and amantadine, as well as the amine precursor L-DOPA, not only stimulated locomotor activity and desynchronized ECoG, but also increased the acetylcholine output from the cerebral cortex of rabbits, cats, guinea pigs and rats (Beani et al, 1968, Beani & Beanchi, 1973, Mantovani et al, 1977, Pepeu & Bartolini, 1968).

It is well known that the ascending reticular pathways from the brainstem contain acetylcholine and are cholinergic (see Shute & Lewis, 1967). It has been shown that administration of the anti-cholinergic drug, hyoscine, had no effect on spike frequency of epileptic foci and did not modify the motor epileptic manifestations in cobalt-implanted rats (McQueen, J. - personal communication). Hyoscine blocks cholinergic transmission in autonomic and central nervous systems. However, it will not eliminate the effect of motor movement on cortical activity, Thus it seems that cholinergic stimulation such as that induced by activation of ARAS is not the only factor in desynchronizing the epileptic ECoG in the cobalt model. The lack of effect of hyoscine is in contrast to the potentiating action of pimozide on epileptic activity, seen in the present work. An enhancement of the frequency of spiking from epileptic foci is not uncommonly seen during spontaneous bouts of drowsiness or drug-induced sedation (Meldrum & Belzamo, 1972). However,

the changes after pimozide were more intense and persistent than those interposed by spontaneous bouts of vigilance in the normal or control rats.

Furthermore, there is no definite evidence as to the amine responsible for stimulation of behaviour and ECoG desynchronization associated generally with cholinergic activation (Phillis, 1968, Jasper & Tessier, 1971). Some workers have stressed the role of noradrenaline (Randrup & Munkvad, 1968, Bliss & Ailion, 1971, Reiss et al, 1970). In contrast, others have claimed the involvement of dopamine (Van Rossum, 1964, Everett, 1970, Costa et al, 1972, Schlechter & Butcher, 1972). In agreement with the latter hypothesis, it has been shown that drugs which increased dopamine to noradrenaline ratio in brain concomittantly increased acetylcholine output, while the opposite changes in this ratio reduced it (Beani & Bianchi, 1970).

Thus, it seems that dopaminergic activity is an important factor in controlling the excitability of cortical neurons. The alteration in epileptic activity of cortical foci induced by dopamine agonists and antagonists seen in the present work confirm this point.

#### Which dopamine system?

It is clear from the present work that dopamine receptor stimulants inhibited epileptic activity and desynchronized the cortical neurons. This effect was dose-dependent with different drugs used. This would suggest that different degrees of stimulation of a dopaminergic mechanism at a particular site is essential in inhibition of cortical hyper-excitability. Possible sites of action remain to be elucidated whether it

is the dopamine receptors in the cortex or other parts of the dopamine system.

Substantial biochemical evidence were provided for the existence of dopaminergic nerve terminals in the cerebral cortex of the rat and cat (Thierry et al, 1972, 1973). These findings were supported by fluorescence, histochemical, and pharmacological observations (Hokfelt et al, 1974, Lidbrink et al, 1974). The increased sensitivity of the glyoxylic acid method of fluorescence histochemistry allowed visualization of characteristic network of dopamine containing nerve terminals ramifying in areas outside the extra pyramidal system including the frontal and anterior limbic cortices of the rat brain (Lindvall & Bjorklund, 1974). Such terminals are found in dorsomedial frontal cortex, anterior cingulate cortex, the ventral part of entorhinal cortex and in the pyriform cortex. The results of lesion experiments showed that the projection to the frontal cortex originates from A 10 cell area of the ventral tegmentum, whereas the projection to the anterior cingulate cortex arises from A 9 cell areas in the lateral substantia-nigra (Lindvahl et al, 1974). However, dopamine terminals have not been found in the motor area of the frontal cortex where both primary and secondary foci are induced in cobalt-implanted rats (Emson & Koob, 1977). Thus, it seems unlikely that dopamine agonists exerted their effect via stimulation of cortical dopamine receptors.

It is well known that the main site of action of these drugs is the dopamine receptors in the striatum. Thus, besides stimulating locomotion and increasing arousal which play at least a part in the desynchronizing effect of these drugs on the cortex, as described before

the question still remains whether the striatum does contribute in controlling motor movements initiated by the motor cortex as well as desynchronizing any epileptic tendency of the cortex. In this respect, it might be worth reviewing some of the fiber connections in the striatum:-

# 1. Input system of the striatum

It has been shown that the striatum receives strong projection from the neocortex, intralaminar nuclei of thalamus, and substantia nigra. Experimental evidence also indicates that the striatum is also the recipient of fibers from the reticular formation and one of the raphe nuclei. As regards the cerebellar input into the striatal circuitry, it has been shown that a larger number of fibers, originating from the dentate nucleus, leaves the cerebellum via the brachium conjunctivum, then decussate in mid-brain and are distributed profusely to the anterior and lateral central thalamic nuclei, which are the centres for termination of the most important outflow channel of the striopallidum (see below). Experimental evidence has shown that areas of termination of the dentato-thalamic and pallido-thalamic projections overlap extensively (Carpenter et al, 1976, Mehler & Nauta, 1974). This cerebellar projection may be considered as forming part of a circuit including the following centers: Cerebral cortex-pontine nuclei-cerebellar cortex-dentate nucleus-thalamus-motor cortex. This cerebro-cerebellar loop shares its thalamo-cortical loop with the principal striatal circuit (see below).



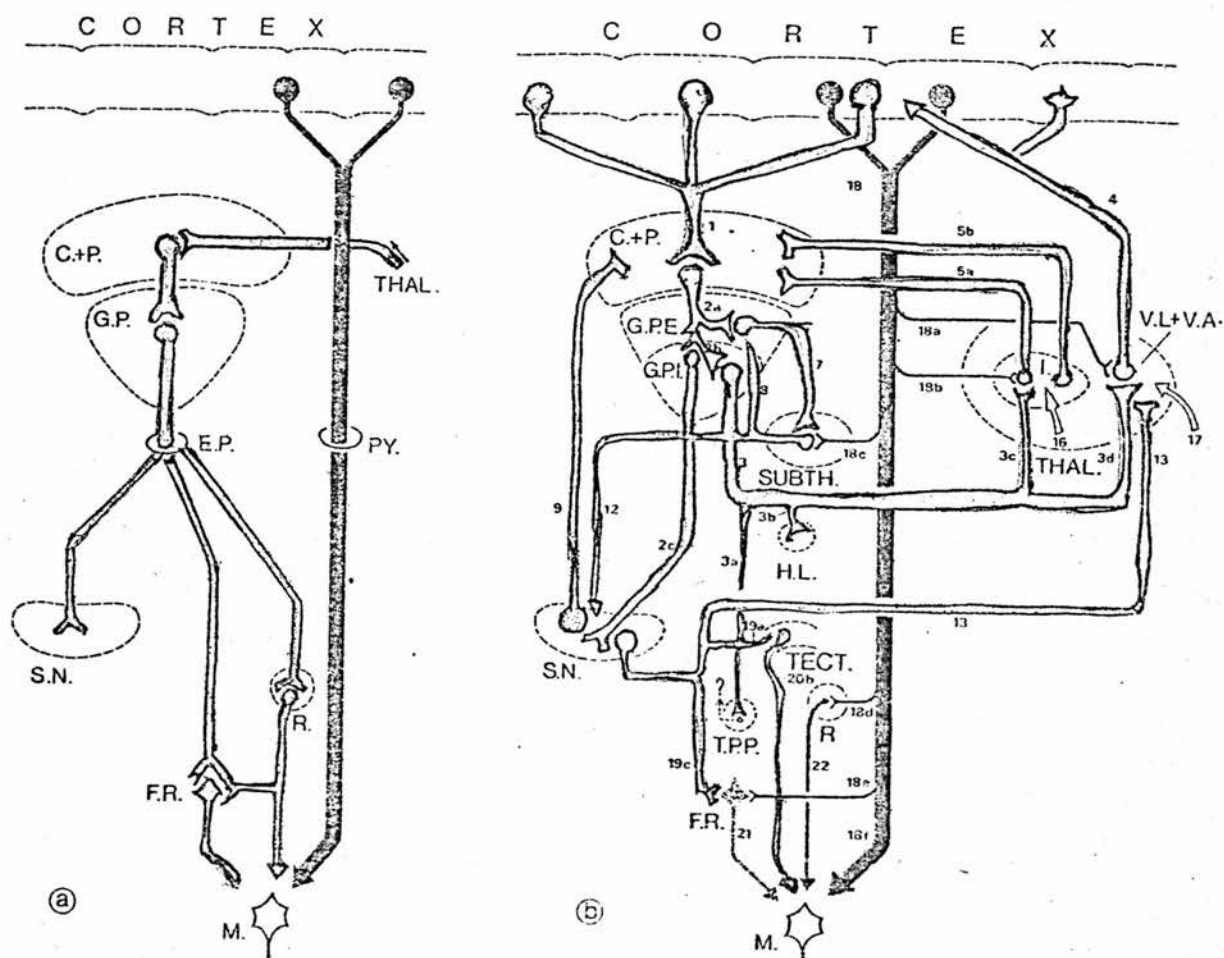
Thus, it seems that the thalamus is an important relay station, linking the activity of the motor cortex with the coordinating action exerted by the cerebellum, as well as with the striatum. This might explain the therapeutic effect of cerebellar stimulation on suppressing certain mild types of epilepsy and cortical desynchronization (Wagner et al, 1975, Cook & Snider, 1955), through the ARAS, partly by altering the phasic inhibition of the thalamus (Yingling & Skinner, 1976).

## 2. Output system from the striatum

(a) The principal striatal circuit (cerebral cortex-striatum-globus pallidus-thalamus-cerebral cortex). (Fig. 29).

This is the most important of all these connections. Studies employing axon degeneration techniques have shown that the whole cortex sends fibers to the caudate-putamen of each site and that all parts of the striatum receive fibers from cortical neurons. The arrangement of this corticostriate projection is on a simple topographical basis, although there is overlap in the termination of fibers from different cortical areas (Webster, 1965, Kemp & Powel, 1970). This means that no part of the striatum is under the influence of one structural or functional cortical area. However, the projection from the sensory cortex to the striatum is particularly large, whereas that of the visual cortex is small. Furthermore, it has been shown that the sensorimotor cortex and its supplementary areas project bilaterally to the striatum (Kunzle, 1975). More recently, it has been shown that about 35% of [ $^3\text{H}$ ] haloperidol





**Fig 29** Diagrams summarizing (a) the classical concept of the pyramidal (PY.) and extrapyramidal (E.P.) motor systems, and (b) our present-day knowledge of the fiber connections of the motor cortex and the striatum. Abbreviations: C., nucleus caudatus; F.R., formatio reticularis; C.P., globus pallidus; G.P.E., globus pallidus, pars externa; G.P.I., globus pallidus, pars interna; H.L., laterat habenular nucleus; I., intralaminar nuclei; M., motor neuron; P., putamen; R., nucleus ruber; S.N., substantia nigra; SUBTH., corpus subthalamicum; TECT., tectum mesencephali; THAL., thalamus; T.P.P., nucleus tegmenti pedunculopontinus, pars compacta; V.A., nucleus ventralis anterior; V.L., nucleus ventralis lateralis.

binding of the striatum is localized to presynaptic dopamine receptors in these cortical terminals (Snyder et al, 1978), and it was suggested that these afferents might be relevant, at least in part, in the mechanism of action of the anti-psychotic dopamine receptor antagonists. Efferent fibers from the striatum converge towards the globus pallidus. The pallido-thalamic projection originates from neurons of the internal pallidal segment to end in the lateral and anterior ventral thalamic nuclei. The lateral nucleus is connected via the thalamo-cortical fibers with the motor area of the cortex from which the pyramidal tract originates. In addition, one striatum is in connection with the other, via fibers of the striopallidal and pallidothalamic projection (Powell & Cowan, 1956, Mehler, 1966, Jones & Levitt, 1974). The existence of this major circuit between the striatum and the cortex suggests that the information derived from the cortex is processed in the striatum, before being fed back via the globus pallidus and thalamus to the motor and premotor areas of the cerebral cortex, thus modulating its activity.

(b) Pallido habenular fibers

The globus pallidus projects upon the lateral habenular nucleus. It is via these fibers that the striatum gain access to the limbic system (Nauta, 1974).

(c) Nigro tectal and nigro reticular fibers connect the striatum with the reticular formation and the superior colliculus from which the reticulo spinal and tecto spinal descending spinal tracts originate, respectively. Thus, the striatum

seems to exert its motor regulatory influence by way of both ascending and descending projection.

Based on these anatomical relations of the striatum and their functional importance, as well as the known pharmacology of dopamine agonists, it was thought feasible for the striatum to affect cortical desynchrony as part of its regulatory action on motor activity, as well as on the excitability of cortical neurons.

The application of the cholinergic drug, carbachol, into the caudate nuclei of the rhesus or squirrel monkey resulted in the appearance of secondary generalized seizures which were blocked by atropine (Cools et al, 1975, Murphey & Dill, 1972). These observations strengthen the classical hypothesis that subcortical structures such as the basal ganglia are important for the generalization and propagation of epileptic attacks (Gestaut & Fischler-Williams, 1959).

On the other hand, the caudate nucleus inhibits bioelectrical epileptiform activity in several brain regions including the cerebral cortex (Amato et al, 1964, Krauthamer, 1963) and amygdaloid nucleus complex (La Grutta et al, 1971), which are characterized by having a low threshold to epileptic phenomena (Feinstein et al, 1970, Ajmon-Marsan & Abraham, 1964). Micro injections of dopamine into the caudate nucleus inhibited epileptic activity in the amygdala induced by the local application of picrotoxin (Stach, 1976). Furthermore, epileptic activity in mirror cortical foci induced by ouabain was inhibited by dopamine and potentiated by haloperidol injected into the rabbit striatum (Stach & Kacz, 1976). The intrastriatal injection of dopamine completely inhibited carbachol-induced generalized seizure in rhesus

monkeys (Cools et al, 1975). It has also been reported that the systemic administration of L-DOPA, with a decarboxylase inhibitor, suppressed the epileptic activity in penicillin-induced (Kobayashi et al, 1976), and cobalt-induced (Scuvee-Moreau et al, 1977), cortical foci. It is also well known that andiogenic, electroshock-induced, and photomyoclonic seizures are modulated by dopaminergic compounds (Billet et al, 1970., Boggan & Seiden, 1971, Killam, 1973, Meldrum et al, 1975).

In the present experiments, the bilateral intrastriatal injection of dopamine (25 µg) reduced rapidly the epileptic activity of cortical foci in the cobalt model. However, the effect was short-lived, lasting for about 45 min. It is possible that a large amount of the injected dopamine was eliminated by the catabolising enzymes catechol-O-methyl-transferase and monoamine oxidase, or by diffusion leaving a portion to reach its site of action at the synapses in the striatum, thus producing this short-lived effect. Similar results were obtained with bilateral injection of apomorphine (60 µg) into each striatum. However, the anti-epileptic effect of apomorphine was more intense than that of dopamine. It has been shown that apomorphine is the most potent dopamine agonist in reducing [<sup>3</sup>H] dopamine binding ( $IC_{50} = 6$  nM) while dopamine is about 30% as active, ( $IC_{50} = 22$  nM). However, like dopamine, the anti-epileptic effect of apomorphine was short-lived lasting about 50 min, and this reflects the rapid metabolism, inactivation and elimination of apomorphine.

These experiments do suggest that stimulation of dopamine receptors in the striatum is a primary event in the action of dopamine agonists in inhibiting cortical epileptic activity.

Furthermore, the unilateral injection of dopamine (25 µg) into the

right striatum caused inhibition of epileptic activity in both the primary and secondary cortical foci. This could imply that fiber connections between the striatum on both sides via pallidum and thalamus remain an important inter-dependent link whereby the primary focus influences the activity of the secondary focus and vice versa. It is possible that dopamine could have diffused to the other striatum to induce suppression of epileptic activity in the secondary focus. This might have caused dissociation of the onset of suppression of epileptic activity on both sides, whereby it would have occurred earlier on the injected side. However, in this experiment both foci were suppressed rapidly and at a similar time after the injection, which might rule out diffusion of dopamine to the opposite striatum <sup>in</sup> /initiating the suppression of epileptic activity on the contralateral side.

The anatomical spread of seizures through the brain depends on the specific site of cortical discharge, the strength and duration of a stimulus, and whether the event is new or chronic. With direct cortical stimulation or the application of topical convulsants, the earliest signals are detected in the contralateral homotopic cortex and homolateral subcortical nuclei, particularly the thalamus and caudate-putamen (Kriendler, 1965, Scheibel & Scheibel, 1966). This reflects spread by specific corticofugal pathways, through the corpus collosum to the homotopic cortex, and by internal capsule to the striatum and the thalamus.

It has already been shown that sectioning of the corpus collosum if carried out within 4 days, prevented the development of secondary focus in the cobalt-implanted rat, but had no effect at later times (Ashcroft et al, 1974). This suggests that the secondary focus becomes independent

of its callosal connections with the primary focus after four days of cobalt application but remains partly dependent upon it through other pathways, like the one linking the striatum on both sides with the thalamus as shown in the experiments described above. Recent experimental work has emphasized both the medial thalamus-frontal cortex and mid-brain reticular formation as important in the mechanism of bilateral seizure spread, the latter being activated early from foci in motor cortex (Kriendler, 1965). Other workers using autoradiographic tracer methods in penicillin-induced foci, suggested that bilateral seizures develop from a unilateral focus primarily through increasing activation of intrathalamic circuits (Collins et al, 1976, Collins & Plum, 1975). In cobalt-implanted rats, contralateral forelimb twitches were evident and very rarely did the animal develop bilateral or generalized seizure. However, thalamic spreading depression suppressed the discharge produced by cortical or hippocampal stimulation in rats (Aquino-Cias & Bures, 1967). Moreover, thalamic lesions have been used as a therapeutic technique in patients suffering from intractable seizures (Mullan et al, 1967, Williams, 1965).

The effect of intrastriatal injection of dopamine agonists demanded looking at the effects of destroying dopamine terminals in the striatum upon cortical epileptic foci.

The bilateral injection of 6-OHDA (20  $\mu$ g) into each striatum caused a significant reduction in the concentration of both catecholamines, in comparison to controls. However, dopamine concentration was more profoundly reduced than that of noradrenaline, reaching to 10% and 15% of control values on the right and left sides, respectively. This effect was accompanied by potentiation of the development of epileptic activity in cortical foci, thus adding more support to the assumption that the striatum exerts an important inhibitory role tending to desynchronize cortical neurons. However, if striatal dopamine was the sole modulator of the activity of cortical neurons, via the major striatal circuit described before, the increased epileptic activity following 6-OHDA lesion of striatum would have been much more pronounced. Thus, an additional factor could augment such inhibition by the striatum upon the cortex. It has been shown that some gabbaminergic neurons in the striatum are not functionally related to the striato-nigral system, but project to the prefrontal cortex (Divac, 1968). The intrastriatal injection of gamma-aminobutyric acid (GABA) was shown initially to potentiate transiently and then to inhibit the epileptic activity of cortical mirror foci induced by ouabain (Stach & Kacz, 1976). The role of GABA in epilepsy has been extensively investigated (Meldrum et al, 1975, Woods, 1975). It has been shown that inhibition of GABA synthesis with consequent reduction in brain GABA concentration lowered the threshold of animals for convulsions. However, treatments that elevated brain GABA are not always anti-convulsant. Amino-oxyacetic acid, an inhibitor of the enzyme metabolizing GABA (GABA-transaminase) has a definite anti-convulsant action at doses of 5-20 mg/kg, while at higher doses it might have a definite convulsant effect (Woods & Pesker, 1973). Moreover, sodium-n-dipropyl acetate is known to elevate brain GABA and



is used in the treatment of epilepsy (Jeavons & Clark, 1974), yet this drug was shown to have no effect on cortical epileptic activity in acute and chronic experiments (Emson, 1976). Thus the anti-convulsant action of these drugs is unlikely to be mediated by elevation of brain GABA levels.

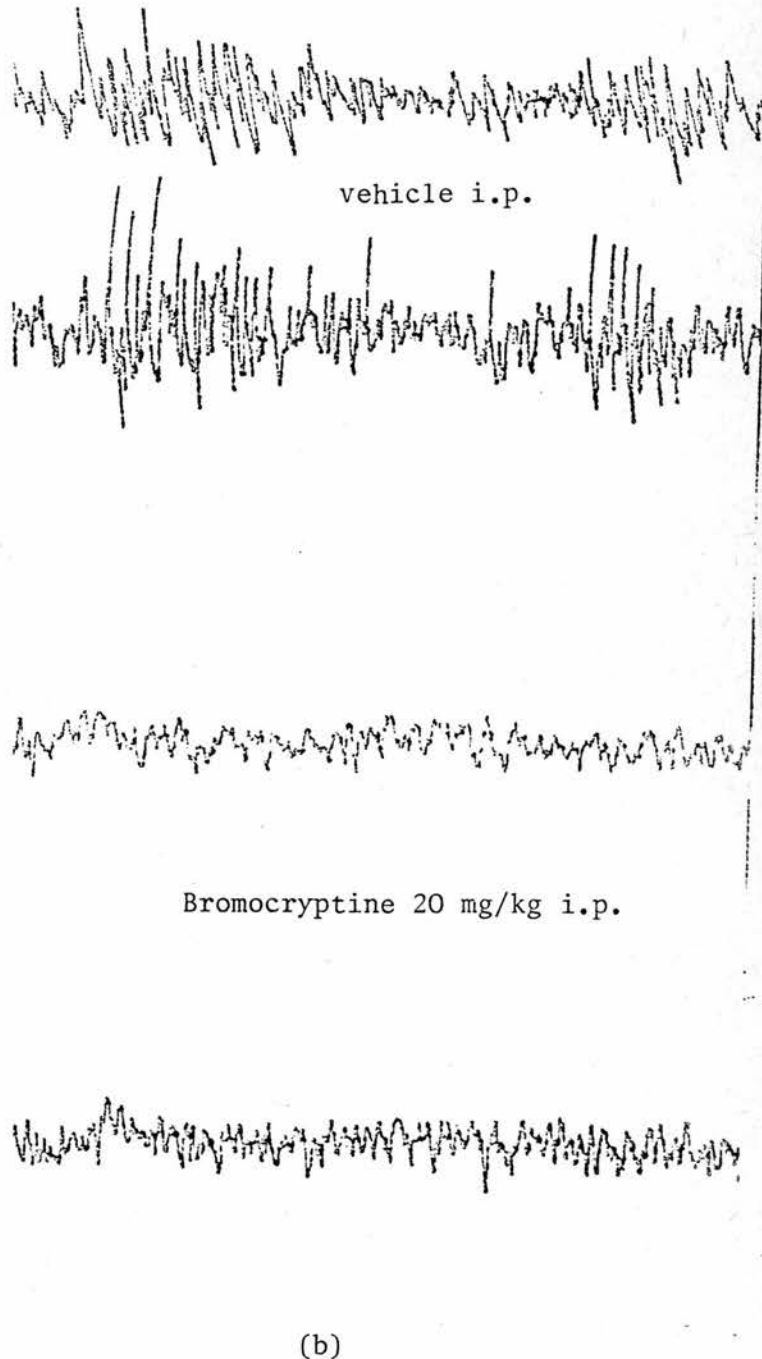
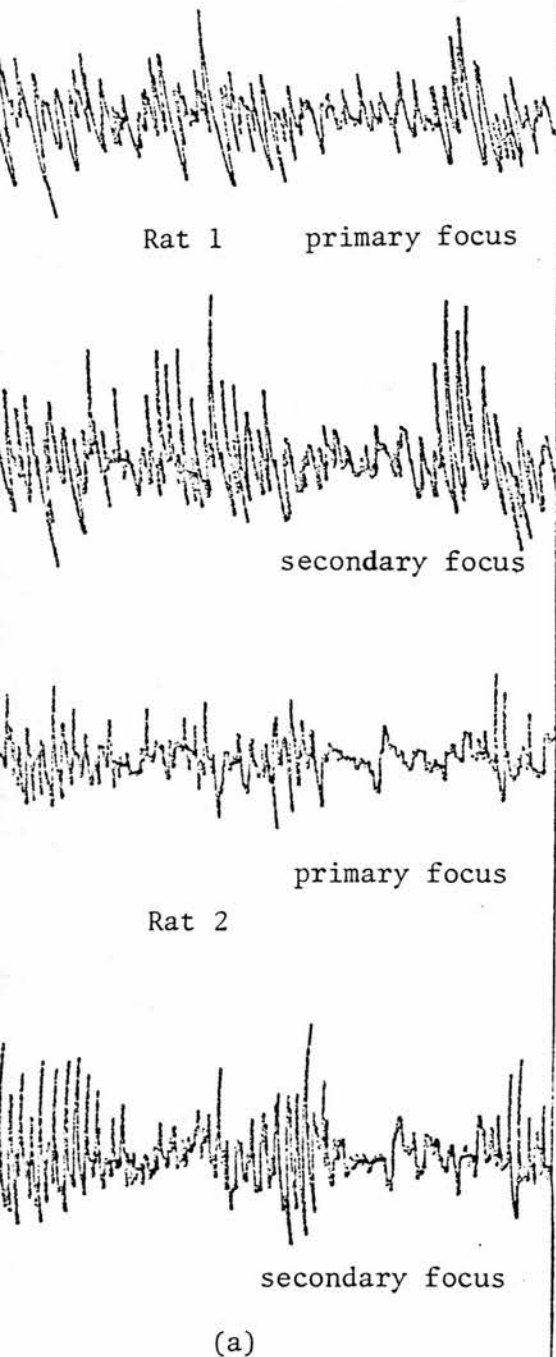
Thus, it seems that the dopaminergic mechanisms in the striatum play an important role in modulating the activity of cortical neurons, tending to desynchronize any tendency to epileptic activity.

In conclusion, it could be said that dopamine agonists exerted their desynchronizing effect on cortical foci at least in part, by stimulation of dopamine receptors in the striatum as well as augmenting activity of ARAS as induced by increased arousal and motor enhancement. Some anti-convulsants, like phenytoin and phenobarbitone have been shown to increase HVA levels in the CSF of treated patients (Corrodi et al, 1967, Chase et al, 1969). Amphetamines which release catecholamines from their nerve terminals, have been used clinically in the treatment of epilepsy, including petit mal. Furthermore, they have been reported to enhance the anti-convulsant action of phenobarbitone and phenytoin in the mouse electroshock model (Rudzik & Johnson, 1970). Thus, it is possible that stimulation of dopaminergic mechanism could explain in part the anti-convulsant action of these drugs.



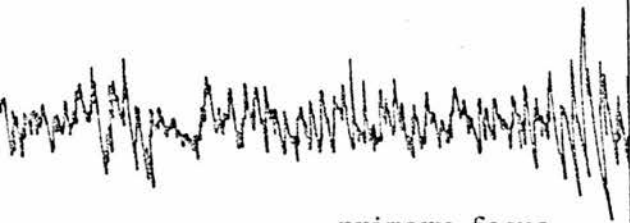
### SECTION 3.

DOPAMINE AND AMYGDALOID KINDLING  
(SEIZURES INDUCED BY SENSITIZATION  
WITH LOW INTENSITY ELECTRICAL  
STIMULATION OF THE AMYGDALA).



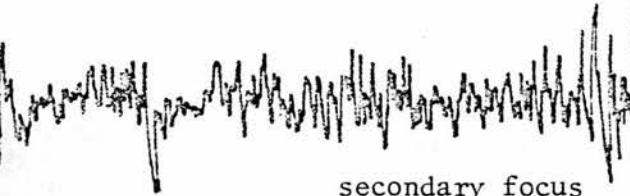
ECOG Record 1: showing the suppressant effect of bromocryptine on the epileptic activity in cobalt-implanted rats.

- (a) A sample of recording during the initial control period of 30 min.
- (b) A sample of recording from the same animals 4 hrs after the intraperitoneal administration of bromocryptine (20 mg/kg) to Rat 2. Rat 1 received an equivalent volume of vehicle, i.p.

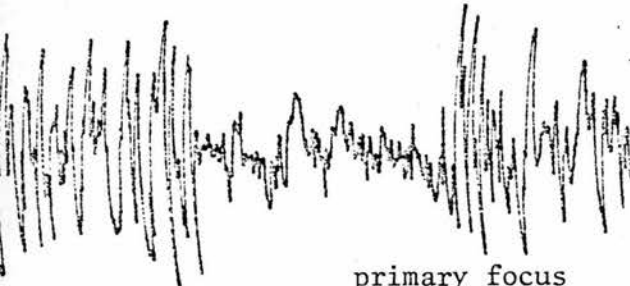


primary focus

Rat 1.

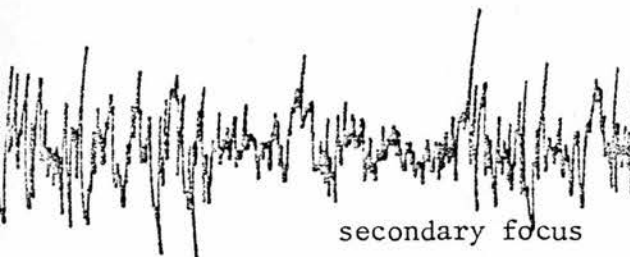


secondary focus



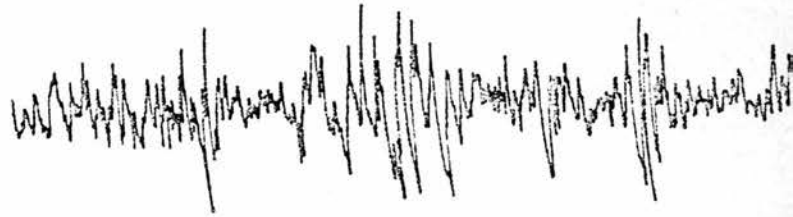
primary focus

Rat 2.



secondary focus

(a)

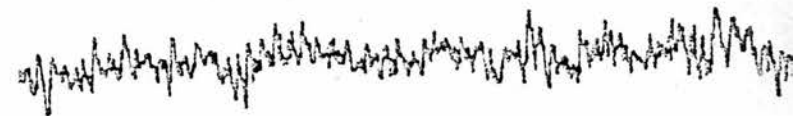


primary focus

normal saline i.p.



secondary focus



primary focus

Lisuride 0.1 mg/kg i.p.

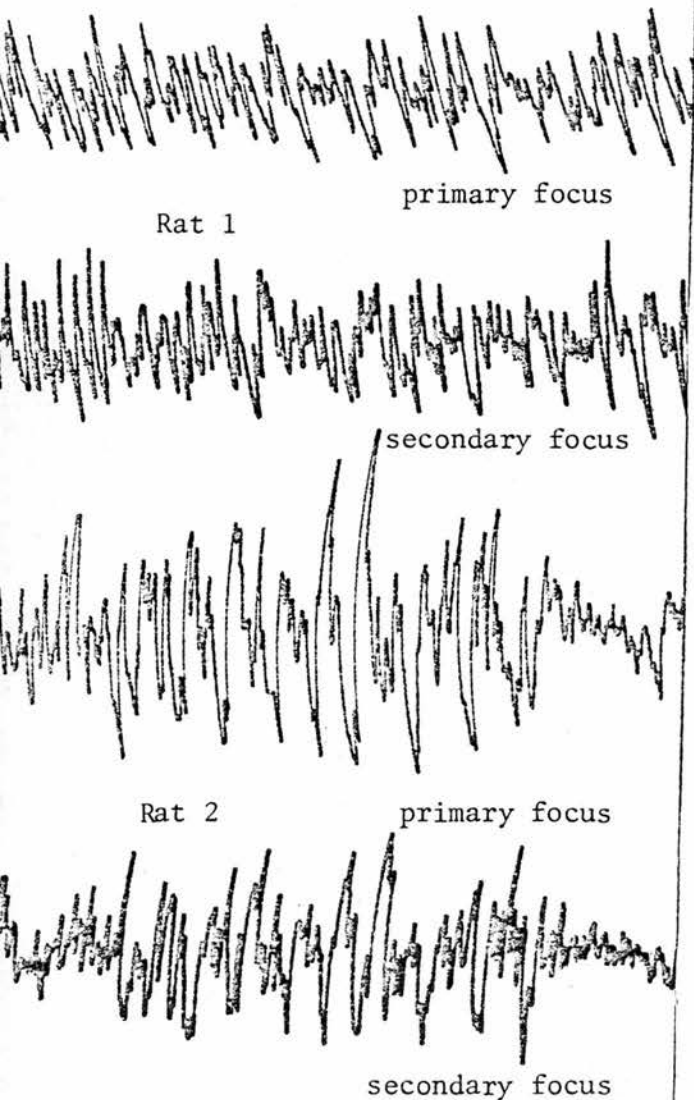


secondary focus

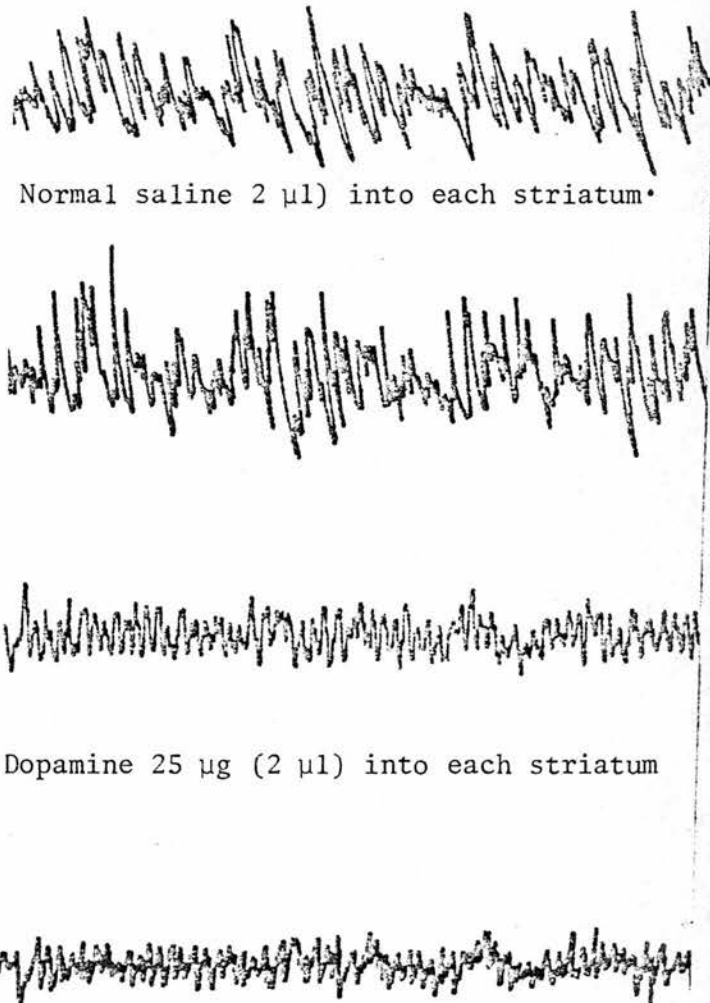
(b)

ECOG Record 2: showing the suppressant effect of lisuride on cortical epileptic activity in cobalt-implanted rats.

- (a) A sample of recording during the initial control period of 30 min.
- (b) A sample of recording from the same animal, 20 min after the intraperitoneal administration of lisuride (0.1 mg/kg) to rat 2. Rat 1 received an equivalent volume of normal saline, i.p.



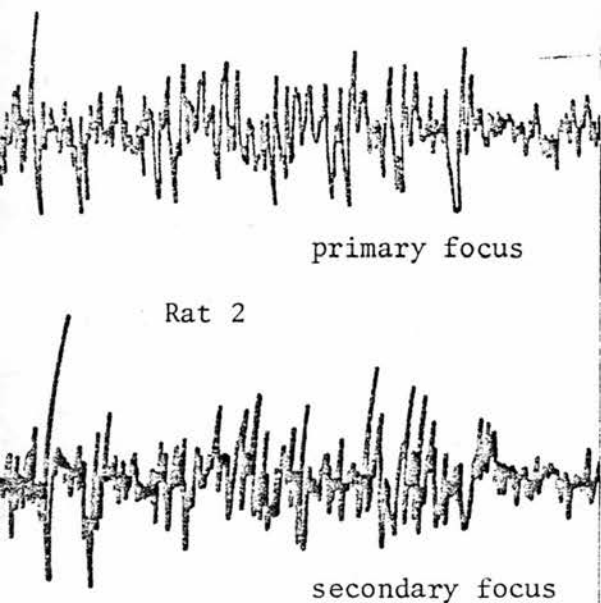
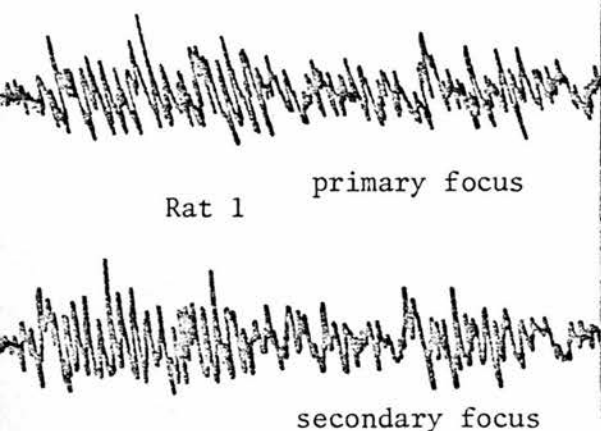
(a)



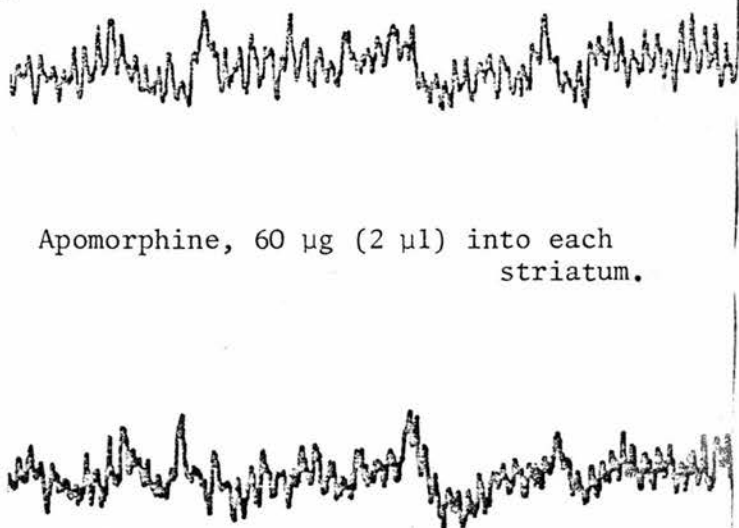
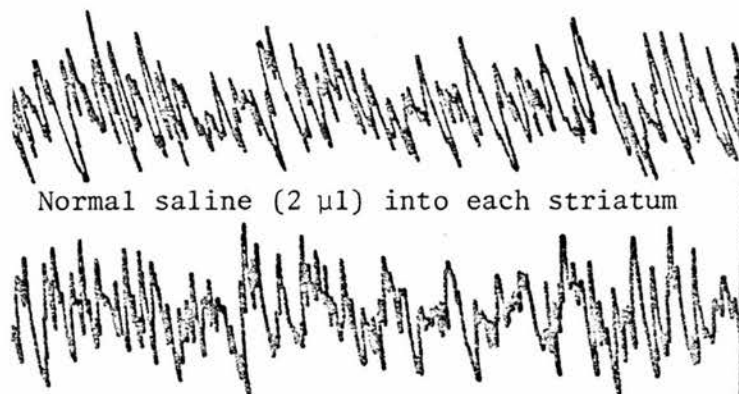
(b)

ECOG Record 3: showing the suppressant effect of dopamine administration into each striatum on cortical epileptic activity in cobalt-implanted rats.

- (a) A sample of recording during the initial control period of 20 min.
- (b) A sample of recording from the same animals 15 min after dopamine injection (2  $\mu$ l) into each striatum of rat 2. Rat 1 received 2  $\mu$ l normal saline.



(a)



(b)

ECOG Record 4: showing the suppressant effect of apomorphine administration into each striatum on epileptic activity in cobalt-implanted rats.

- (a) A sample of recording during the initial control period of 20 min.
- (b) A sample of recording from the same animals after the administration of apomorphine (60  $\mu$ g) into each striatum of rat 2. Rat 1 received 2  $\mu$ l normal saline.

#### A. DEVELOPMENT AND MECHANISM OF KINDLING

Periodic bipolar stimulation of a number of sites throughout the olfactory-limbic system can lead to grand mal development and intensification of behavioural convulsions (kindling) in a variety of species (Goddard, McIntyre & Leech, 1969), even at current intensities that are initially too low to produce any behavioural or electrographic effect. The most productive areas in kindling phenomenon involves deep limbic structures in rats (Goddard, 1967, Racine, 1972a and b), cats (Goddard, 1969, Morrel, 1973, Wada, 1974), rhesus monkeys (Wada, 1974) and Baboons (Wada & Osawa, 1974). For example, if the rat amygdala is periodically stimulated at a level which produces neither electrographic nor behavioural effects, eventually the afterdischarge threshold may be reduced to the point where subsequent stimulations reliably elicit afterdischarge. If stimulations are then continued, mild motor automatism may appear which increases in severity with each successive stimulation until motor seizure characterized by facial and forelimb clonus, weeping and loss of equilibrium can be elicited reliably (Racine, 1972a).

Although kindling can be produced from a wide variety of olfactory, limbic and cortical areas, the number of stimulations necessary to kindly full motor seizures is a function of the site of stimulation (Goddard et al, 1969). However, since the amygdala has been found to be particularly responsive, most studies on kindling have employed amygdaloid stimulation. Regardless of the level of responsiveness of any particular structure, however, kindling cannot be produced unless stimulations are distributed

over a time. Goddard et al (1969), found the number of stimulations leading to kindled convulsions to be inversely related to the duration of the interval between stimulations. It was difficult to kindle rats with interstimulation intervals of less than 20 min, and interstimulation intervals of 24 hrs or longer were found to be optimal. In a subsequent study using different stimulation parameters, Racine et al (1973) confirmed the general inverse relation between the duration of the interstimulation interval and the number of stimulations required to kindle full motor seizure, but found that intervals as short as one hour could be used without significantly retarding the rate of kindling. Moreover, they found that kindling and not just the expression of motor seizures was blocked at short intervals, massed stimulations (15 stimulations administered at 15 min intervals) produced only a slight reduction in the number of distributed stimulations later required to kindle full motor seizures.

Racine (1972a) has provided convincing evidence that the elicitation of afterdischarge at the site of stimulation is a necessary prerequisite for the kindling of motor seizures. Periodic stimulation maintained at a level below the afterdischarge threshold did not lead to the development of motor seizure, nor did it reduce the number of suprathreshold stimulations, later required for kindling. Moreover, the main electrophysiological correlate of the kindling process appears to be the degree to which the afterdischarges generalise from the site of stimulation to other neural structures (Racine et al, 1972). During the course of kindling, spike amplitudes in secondary foci progress from very small or non-existent to amplitudes as great as those in the primary focus (Racine, 1972a).



Racine (1972b) has also reported that an appreciable reduction in the afterdischarge threshold can occur during kindling, but at least three lines of evidence suggest that the reduction in the afterdischarge threshold is independent of the kindling which can occur concomitantly. First, periodic amygdaloid stimulation maintained below the afterdischarge threshold reduces the afterdischarge threshold without leading to the development of motor seizure (Racine, 1972a). Secondly, stimulation of the posterior neocortex does not kindle motor seizures even though it reduces the afterdischarge threshold (Racine, 1975). And, thirdly, the reduction of afterdischarge threshold appears to be restricted to the site of stimulation while kindling of one brain site greatly influences the degree to which other brain sites can be kindled (Goddard et al, 1969, Racine, 1972b, Racine 1975, McIntyre & Goddard, 1973).

Perhaps the most important feature of the change in neural function induced by periodic brain stimulation is that they are enduring, if not altogether permanent. Goddard et al (1969) rekindled rats after 12 weeks of stimulation-free interval and found a saving of 90% in the number of stimulations required to elicit a full motor seizure. Similarly, Racine (1972b) found that the decrease in afterdischarge threshold produced in rats by repeated hippocampal or amygdaloid stimulation persisted at least 6 weeks after all stimulation were curtailed.

Although the permanence of the change in motor seizures are more striking, the changes in afterdischarge threshold are particularly important since they can be produced by subthreshold stimulation. Even stimulation levels which produce no obvious electrographic or behavioural change can produce a lasting increase in the susceptibility of the brain to later stimulations.



The evidence suggests that the kindling effect does not result from a localized histological change at the stimulated site, but depends widely upon disseminated neuro-circuits being constructed trans-synaptically (Goddard et al, 1969, Racine et al, 1972, Racine, 1972b, Wada & Sato, 1974, Wada et al, 1974). This view is further supported by the 'transference' phenomenon, which is the efficient rekindling in a secondary brain structure after establishing the kindling effect in the primary structure. Moreover, permanency of behavioural modification and of electrographic seizure discharge was confirmed in kindled animals (Goddard, 1972, McIntyre & Molino, 1972, Wada et al, 1974). These findings strongly suggest that the kindling procedure can lead to secondary functional alterations and predictable organisation of brain function, culminating in a permanent state of epileptogenic activity that is strikingly similar to human epilepsy.

A number of reports suggested that the mesencephalic reticular formation and related structures in mid brain and lower brain stem may play a prominent role in the spread of afterdischarges in amygdaloid kindling (McCaughran et al, 1975, McIntyre, 1975, Wada & Sato, 1974, 1975). Studies of amygdaloid kindling in rats, cats, rabbits and baboons (Wada, Sato & McCaughran, 1974, Tanaka, 1972) showed progressive involvement of basal cortical areas as well as the development of spike discharge in the frontal cortex, associated with complex seizure patterns. As kindling progressed, the cortical epileptiform activity became independent of the amygdaloid afterdischarge. Furthermore, studies employing bilateral aspiration lesions of frontal cortical areas, particularly the prefrontal and orbital cortex, have shown retardation of the rate of kindling. Nevertheless, kindling occurred in lesioned rats, indicating that these

cortical areas are not essential for the development of amygdaloid seizures (Corcoran et al, 1975).

B. INNERVATION OF THE AMYGDALA

Cholinergic input

The cholinergic nerve terminals in the amygdala originate from the ascending cholinergic reticular system (Shute & Lewis, 1967). Furthermore, biochemical and histochemical evidence suggest that the basolateral nuclei contain more cholinergic nerve terminals than the centro medial nuclei of the amygdala.

Catecholaminergic input

Noradrenergic terminals have been demonstrated in the amygdala by fluorescence histochemistry (Fuxe, 1965, Ungerstedt, 1971). Lesion and radiographic studies indicate that at least a portion of these terminals originate from the locus coeruleus and reach the amygdala via the dorsal noradrenergic bundle and stria terminalis (Segal et al, 1973, Ungerstedt, 1971).

The dopaminergic input to the amygdala originate from the substantia nigra and passed through the internal capsule and stria terminalis (Ungerstedt, 1971). The highest tyrosine hydroxylase activity and catecholamine concentrations in the amygdala are located in the central nucleus (Fuxe, 1965, Ben-Ari et al, 1975). Furthermore, a higher tyrosine hydroxylase activity and catecholamine level were found in the basolateral than in cortico-medial areas (Ben-Ari et al, 1975), thus supporting the findings using fluorescence histochemistry studies of the amygdala

(Hokfelt et al, 1974). Furthermore, regional differences within the amygdala were much larger for dopamine than for noradrenaline (Ben Ari et al, 1975).

#### Neural output from the amygdala

The fiber connection of the amygdaloid complex comprises connections with the following:-

- (a) the olfactory area
- (b) the neocortex
- (c) the lateral preoptic area and hypothalamus
- (d) the dorsal thalamus.

These connections are thus important in the spread of afterdischarge initiated in the amygdala and, consequently, in the generalization of subsequent seizure.

#### C. CLINICAL AND EXPERIMENTAL IMPLICATIONS OF KINDLING

The utility of the kindling phenomenon as a model of epileptogenesis was the first clinical application to be emphasized (Goddard et al, 1969, Morrel, 1973). There are striking parallels between the kindling phenomenon and the progressive development of epileptic symptoms in human patients. In untreated clinical cases as in kindled animals, there is often a progressive development of symptoms within a particular attack as well as from one fit to the next (Huglings-Jackson, 1870). By definition, human epilepsy is a spontaneously recurring, self-sustained, paroxysmal dysfunction of the brain, but early efforts to kindle rats to the point

where they would display such spontaneous seizures did not meet with success (Goddard et al, 1969). However, further experiments have clearly demonstrated that periodic amygdaloid stimulation will eventually result in the development of recurrent spontaneous seizures in both rats (Pinal et al, 1973) and cats (Wada & Sato, 1973, Wada, Soto & Corcorann, 1974).

In the rat the production of spontaneous seizures simply appears to be a matter of extending the number of periodic brain stimulations. If amygdaloid stimulations are continued beyond the point where most kindling experiments are curtailed, there is a gradual reduction in the day-to-day variability of the class 5 motor seizure pattern (Racine, 1972a) followed by the eventual development of two new motor seizure patterns. The first involves multiple rearing and following sequences and the second can best be described as a running fit. It appears that the electrographic basis for the development of spontaneosity is the same for both rats and cats (Pinel, Mucha, Phillips, 1975, Wada et al, 1973). In both cases the development and proliferation of interictal spike discharges appears to be the major electrographic correlate of the epileptogenesis of spontaneous seizures. Now that it has been well established that repeated local brain stimulation will eventually lead to the progressive development of epileptic syndrome, the kindling model should prove to be a valuable tool in the controlled experimental study of factors associated with epileptogenesis.

The second major clinical application of kindling has been to use it as a procedure for assessing the convulsant and anti-convulsant properties of drugs and other stimuli. Babington & Wedeking (1973) studied the effects of a variety of centrally-active agents on kindled seizures elicited from the amygdala or sensori-motor cortex. Once the seizures

were stabilised, drug effects on seizure duration were assessed. Anti-depressive drugs were found to be more effective in suppressing seizures elicited by amygdaloid stimulation than those elicited from the cortex, whereas both anti-anxiety and anti-epileptic drugs exerted non-selective blockade, but the anti-anxiety drugs were more potent. However, the effect of drugs can be assessed not only on each individual seizure, but on the progressive intensification of seizures from one stimulation to the other. By determining the extent to which anti-convulsants are capable of blocking the kindling motor seizure, it may eventually become possible to predict which anti-convulsant will be most successful prophylactically in a clinical situation (Wada, 1975). Wise & Chinerman (1974) reported that diazepam and phenobarbitone, but not diphenylhydantoin, can block the progressive intensification of motor seizures but not the local decrease in afterdischarge threshold produced by repeated electrical stimulation of the amygdala.

Electrical current has been applied to the human brain in an attempt to identify epileptic foci (Adams & Rutkin, 1972), to treat schizophrenia (Carletti, 1956), to change emotional level of psychotic patients (Heath, 1970) and to relieve chronic pain (Heath & Mickle, 1960). However, it was argued whether brain stimulation could leave the organism more susceptible to seizures, not only during the course of treatment (Goddard, 1971), but also to convulsant agents administered long after the electrical stimulation therapy had been curtailed.

It has been shown that alcohol withdrawal can interact with prior brain stimulation to intensify epileptic symptoms (Pinel et al, 1975). Furthermore, withdrawal from barbiturates and many other sedatives or

tranquillizers can produce convulsions similar to those seen in alcohol withdrawal (Itil, 1970). Moreover, repeated amygdaloid stimulation, either above or below the afterdischarge threshold, was found to intensify the subsequent convulsive reaction to metrazol in rats (Pinel, Skelton & Mucha, 1975).

Apart from local brain stimulation which is infrequently employed as a therapeutic measure, kindling can be produced in response to the repeated administration of a variety of convulsive agents. Sub-convulsive doses of metrazol in rats, at 3-day intervals, resulted in the development of minor myoclonic response after four injections, and eventually grand mal seizures were elicited in some animals (Mason & Cooper, 1972). Leech (1971) observed that mice which are initially resistant to audiogenic seizures will eventually become responsive to stimulation if they were exposed to a short bout of audiogenic stimulation daily. Moreover, the daily injection of carbachol into the amygdala, hippocampus or caudate in rats, in sub-convulsive doses will eventually elicit motor seizures (Vosu & Wise, 1975). Repeated electroconvulsive therapy is widely used in psychiatric treatment, especially in depressive illness. The duration of the inter-stimulation interval may influence the susceptibility to electroconvulsive seizures. It has been shown that electroconvulsive stimulations (ECS) administered at intervals of 3 (Ramer & Pinel, 1974) or 7 (Pollack et al, 1963) days, resulted in increased susceptibility and intensification of the induced seizure. However, at interstimulation intervals of 24 hrs or less, ECS series were found to cause a progressive reduction in seizure threshold and severity (Essig et al, 1961, Ramer & Pinel, 1974).

#### D. CATECHOLAMINES AND KINDLING SEIZURE SUSCEPTIBILITY

The role of brain catecholaminergic systems in the neurohumoral basis of seizure susceptibility has been studied by many workers. It was reported that catecholamines are inhibitory to seizures in general (Maynert, 1969). Depletion of central catecholamine levels induced by reserpine or intra-ventricular injection of 6-OHDA has been shown to accelerate kindling (Arnold et al, 1973, Corcoran et al, 1974), while increased catecholamine turnover, as that induced by stress, was found to retard the development of kindling seizure. These findings are consistent with the previous reports that increased seizure susceptibility occurred when brain catecholamine levels were significantly lowered, while decreased susceptibility was found when brain dopamine levels were significantly elevated (Boggan & Seiden, 1971, Bourn et al, 1972, Izumi et al, 1973, Mackenzie & Soroka, 1972). All the above findings suggest that kindled seizure susceptibility may be dependent upon the depletion of brain catecholamine levels. Seizure susceptibility, which is based on marked depletion of both catecholamines, is increased by participation of activated serotonergic system during slow wave sleep and is decreased by the physiologically active catecholaminergic system during REM sleep.

Some workers have shown that marked depletion of catecholamines occurred in both cerebral hemispheres of hippocampal kindled cats (Sato et al, 1975). Other workers, however, reported a reduction in noradrenaline levels in the hypothalamus, but found no change in brain dopamine levels in amygdaloid kindled rats (Callaghan & Schwark, 1975). Using micro-iontophoretic studies it has been shown that dopamine behaves as inhibitory transmitter in the amygdala (McCrea, Jordan & Lake, 1973, Ben-Ari & Kelly, 1974). More recently, it has been shown that unilateral lesion of the stria terminalis, which contains catecholamine inhibitory afferents to the



amygdala, facilitate the development of the early stages of kindling induced by ipsilateral amygdaloid stimulation (Engel & Katzman, 1977). Furthermore, the dopamine content was reported to be locally reduced at the site of the stimulated amygdala in kindled rats (Engel & Sharpless, 1977). These findings suggested that dopamine in the amygdala is inhibitory to the kindling process.

### Tyrosine hydroxylase

Tyrosine hydroxylase activity is the rate-limiting step in the biosynthesis of catecholamines, and the most specific indicator of the functional integrity of catecholaminergic neurons (Levitt et al, 1965, Spector et al, 1965) (For Review, see Introduction, Section 1).

It has been shown that two molecular types of tyrosine hydroxylase exist, controlling the synthesis of noradrenaline or dopamine in their respective neurons, (Reis et al, 1975). Although brain tyrosine hydroxylase is highly localised to catecholaminergic nerve endings, histochemical studies following lesions of catecholamine neurons have shown accumulation of catecholamines proximal to the lesion and their disappearance at the nerve endings (Dahlstrom & Fuxe, 1965). This implies a continuous transport of the enzyme from the cell body to nerve terminal. Nagatsu et al (1971) investigating the enzyme activity in the bovine caudate nucleus were unable to produce a linear increase in L-DOPA production with increasing amounts of tissue unless the enzyme was purified. Using purified tyrosine preparations, Hendry & Iversen (1971) were able to estimate the enzyme activity in small amounts of neural tissue, and their method was quite sensitive. Moreover, brain tyrosine hydroxylase is responsive to slightly different conditions of pH than the adrenal enzyme



the optimal activity occurring at pH 6 (McGeer et al, 1967).

E. AIM OF THE WORK

The present work was done in order to follow on the possible role of the dopamine system in amygdaloid kindling in a manner analogous to the focal cortical foci induced by cobalt already described. In the first part of this work tyrosine hydroxylase activity was estimated in the stimulated amygdala as well as in various brain regions that are involved or could be implicated in the spread of amygdaloid afterdischarge. The second part of the present work utilizes the dopamine agonists as a pharmacological tool in manipulating the possible underlying mechanism in kindling. This work might help in the understanding of the aetiology and spread of seizure activity from subcortical foci such as those initiated in the amygdala in comparison to cortical foci initiated in the frontal cortex.

## METHODS

### A. ANIMALS

During all kindling experiments, male Wistar rats 180-200 g in weight, were used.

### B. DEPTH ELECTRODE

The bipolar electrode was prepared by twisting a suitable length of nichrome wire and cutting it at the tip, allowing a length of 1 cm for depth at implantation. Electrodes were checked for conductivity using an avometer.

### C. OPERATIVE PROCEDURE

#### 1. Anaesthesia

Halothane (Fluothane, ICI) was used with medical air (BOC). 4% halothane was used for induction, and maintained at a concentration of 2%. The flow rate was 2.5 litres/min.

#### 2. Surgical procedure for electrode implantation

Under halothane anaesthesia, rats were implanted with bipolar stimulating electrodes in the basolateral region of their left amygdalae.

A curved cranial incision, 2 - 3 cm long was made in the scalp, extending from the region near the left eye backwards towards the lambdoid suture. After separating the underlying fascia, the skull was exposed. A hole was drilled, using No. 6 size burr, on

each side of the skull 2 mm anterior and lateral to coronal and sagittal sutures, respectively, and then two stainless steel recording electrodes were fitted to these holes, and fixed with acrylic cement(Simplex).

Using the stereotaxic frame a point on the left side of the skull was localized 4.5 mm anterior to the interaural line and 4.4 mm lateral to the midline (which corresponded to the sagittal suture if the rat is properly positioned). At this site, a hole was drilled, using No. 6 burr, and the dura mater incised with the tip of a fine needle. Thus, the brain surface was exposed and viewed in an operation microscope (Zeiss). The bipolar electrode was then implanted at this point 7.9 mm deep to the cortical surface into the basolateral region of the left amygdala (Konig and Klippel, 1963) and fixed to the skull with acrylic cement. Haemostasis was secured and skin closed with silk after spraying the wound with polybactrin.

### 3. Post-operative period

No special post-operative care was necessary. Scalp wounds healed remarkably well. Infection was rarely a problem. The animals were allowed one week for recovery and daily stimulation was begun on the 8th day. During that period, the rats were examined daily, and animals with loose or dislocated electrodes were discarded. After several weeks animals do lose their electrodes presumably as a result of getting them stuck in the wire loops of the cage. However, it was possible to keep some animals in the kindled state with electrodes intact for as long as 5 months after operation.

In all experiments, stimulated rats were kept separate from controls. Sham-operated and unoperated controls were kept separate from each other. Drug-treated rats were kept separate from saline or vehicle-treated controls.

Not more than 4 rats in each group were assigned to a cage. Food and water were allowed ad libitum, except during a period of a few hours associated with the stimulation and recording procedures.

#### D. PARAMETERS OF STIMULATION

Beginning on the 8th post-operative day a suprathreshold constant sinusoidal current ranging between 100-300  $\mu$ A and consisting of 1 msec biphasic square-wave pulse at a frequency of 60 Hz was delivered to the stimulated rats for one sec daily via the stimulating electrode, using a constant current physiological stimulator (Farnell).

Electrical activity from the amygdala was amplified and recorded on a Grass polygraph (model 7) before and after stimulus delivery. The recording paper speed was 10 mm/sec. The duration of the ensuing after-discharge in secs was measured on the recording paper starting from the point of stimulation.

#### E. DEVELOPMENT AND STAGING OF MOTOR SEIZURE

Daily stimulation was continued until the rats developed generalized convulsions after stimulation for 2-3 consecutive days. As the number of stimulations increased, the rats developed progressive seizure manifestation of amygdaloid kindling, starting with facial twitching, progressing to forelimb clonus and finally generalised seizure. This pattern was previously reported by Racine (1972a). A semi-quantitative estimate of

the intensity of seizure was done using the scale described by Racine (1972a) as follows:-

- |         |                                |
|---------|--------------------------------|
| Stage 0 | No seizure                     |
| 1       | Mouth and facial movement      |
| 2       | Head nodding                   |
| 3       | Forelimb clonus                |
| 4       | Rearing (Kangaroo posture)     |
| 5       | Rearing and falling backwards. |

Thus, a full motor seizure with loss of postural control is referred to as class 5 motor seizure. Daily stimulation was continued once daily until the rats developed generalized seizure for 2-3 days. The duration of the evoked afterdischarge was measured, and the number of stimulations required for the development of different stages of seizure were noted for each rat.

Tissue sections with the microtome showed the electrode tracts in the region of the amygdala.

#### F. DESIGN OF EXPERIMENTS

##### 1. DETERMINATION OF AFTERDISCHARGE THRESHOLD DURING DEVELOPMENT OF KINDLING

Twelve rats were used of which only 7 reached the final stages of generalized seizure. Starting on the 8th day the minimal current intensity required to induce afterdischarge was determined daily until the animals developed generalized convulsions for 2 days. The number of stimulations required for the development of different stages of the motor seizure was also noted.

2. TYROSINE HYDROXYLASE ACTIVITY IN VARIOUS BRAIN REGIONS  
IN KINDLED RATS

A. Experimental procedure

Twenty-one male Wistar rats were used in the experiment. Seven of these rats served as unoperated controls. Under halothane anaesthesia, the basolateral region of the left amygdalae of 14 rats were stereotaxically implanted with bipolar stimulating electrodes, as described in the method. Of these animals, 7 were stimulated and 7 served as sham controls.

During the post-operative period and during experimentation, stimulated rats were kept separate from controls.

B. Dissection

Four weeks after the last stimulation, rats were killed by decapitation and their brains were dissected out quickly on a cold stage at 4°C. The following dissection was done with the aid of a dissecting microscope:

The whole hippocampus was carefully dissected out from the overlying cortex on both sides of the brain and was then removed entirely. Two vertical cuts were then made from the inferior surface of the brain, the first at the level of the optic chiasma (A 5500 microns)(Koning & Kippel, 1963) and the second at the level of junction of the cerebral peduncles with the base of the cerebral hemisphere (A 3000 microns), thus dividing the brain into three parts. From the anterior part, tissue portions were taken from the frontal cortex and striatum on both

sides. From the middle part, both amygdalae were dissected out under the dissecting microscope and care was taken not to include the adjacent pyriform cortex in the dissection of the amygdala. Tissue portions were then taken from the thalamus and hypothalamus with the aid of the dissecting microscope. Kindled, implanted and unoperated control rats were treated identically in all phases of sacrifice.

#### Storage of tissue samples

Tissue samples, placed in polythene bags, were stored by freezing in liquid nitrogen ( $-195^{\circ}\text{C}$ ). The duration of storage was about 35 days before being assayed biochemically for tyrosine hydroxylase activity. No significant loss of enzyme activity was found over that period of storage. The enzyme activity in the brain of control unoperated rats, which was stored for that period, showed no greater variation than that in freshly killed rats.

#### Electrode tracts

The dissection described above did not allow looking for electrode tracts. However, the pattern and rate of seizure development and of the elicited afterdischarge strongly indicated that the electrode tips were in the amygdala.

#### C. Biochemical analysis of tyrosinehydroxylase activity

Kindled, implanted and unoperated control rats were treated together in all phases of sacrifice and biochemical analysis.

Homogenates of tissue sample were assayed together in duplicate for tyrosine-3-hydroxylase activity according to the method of Hendry & Iversen (1971). The extract of brain was incubated with [ $^3\text{H}$ ] labelled tyrosine in the presence of an inhibitor of aromatic amino acid decarboxylase. At the end of the incubation period the amount of titrated 3:4-dihydroxyphenylalanine ([ $^3\text{H}$ ] L-DOPA) formed was measured. This is proportional to the level of activity of tyrosine hydroxylase in tissue extracts. The incubation mixture, containing both [ $^3\text{H}$ ]-L-DOPA and [ $^3\text{H}$ ] tyrosine was passed through an alumina column. [ $^3\text{H}$ ] L-DOPA was adsorbed on alumina and thus retained, while [ $^3\text{H}$ ] tyrosine did not adsorb on alumina and was washed out with dilute Tris buffer. The retained [ $^3\text{H}$ ] L-DOPA was eluted from alumina by glacial acetic acid and a sample of the eluate was then taken for [ $^3\text{H}$ ] counting.

#### Homogenization

Homogenization of weighed tissue was done, on samples which were kept frozen on solid  $\text{CO}_2$  ( $-75^\circ\text{C}$ ), with 20 mM potassium phosphate buffer pH 7.4 and containing 0.1% of 1% Triton-X.

For each 1 mg of tissue, 10  $\mu\text{l}$  of buffer was used for homogenization in an Ependorff tube. [ $^3\text{H}$ ] 2:3 side chain L-tyrosine (specific activity 22 Ci/mMol, Radiochemical Center, Amersham) was purified before use by incubation for 30 min with alumina equilibrated with 0.5 M potassium phosphate buffer, pH 7.4. This procedure would remove any [ $^3\text{H}$ ] labelled catechol compounds that may have been present as impurities. Thus 100  $\mu\text{l}$  of the stock [ $^3\text{H}$ ] tyrosine solution was added to a mixture of 100  $\mu\text{l}$  alumina suspension (Neutral grade, Sigma) equilibrated with 0.5 M  $\text{KPO}_4$  buffer, pH 7.4 and 200  $\mu\text{l}$  of 5 mM Tris (Tris-hydroxymethyl melamine).



buffer, pH 8.6 This mixture was shaken continuously for 5 min using a mechanical shaking apparatus and with sufficient force to keep the alumina in suspension. It was then allowed to stand for 30 min. The supernatant was used as the purified tyrosine substrate for the assay of tyrosine hydroxylase.

The final substrate solution for the enzyme activity was prepared by mixing equal volumes of the purified tyrosine solution with 0.2 M potassium phosphate buffer solution containing 2 mM DMPH<sub>4</sub> (2-amino-4 hydroxy-6,7 dimethyl tetrahydropteridine, Roche), 6.25 mM of B-mercaptoethanol and 3.05 nM 3-hydroxybenzyl oxyamine (NSD - 1055, Smith & Nephew), as the aromatic amino acid decarboxylase inhibitor. This concentration of NSD-1055 was reported to produce a complete inhibition of decarboxylase activity. This final substrate solution was used for the reaction and it contained 66 pmol (1  $\mu$ Ci) [<sup>3</sup>H] 2,3 side chain-1-tyrosine in each 10  $\mu$ l solution.

#### Reaction

To start the reaction, 10  $\mu$ l of the substrate solution described above, was added to 10  $\mu$ l of tissue homogenates. The reaction was allowed to proceed for 20 min at 37°C in stoppered Eppendorf tubes and was terminated by the addition of 250  $\mu$ l of 0.4 M perchloric acid containing 2  $\mu$ g/ml of cold L-DOPA carrier.

#### Neutralization

The acid solution was then neutralized to pH 8 by the addition of 4 ml solution containing 0.1 M Tris, 0.2 M sodium EDTA and 0.3 M sodium hydroxide.

### Adsorption of [ $^3\text{H}$ ] L-DOPA

The solution was then passed through 4 x 0.25 cm columns of neutral alumina, equilibrated in potassium phosphate buffer, pH 7.4 and washed with 10 ml of 5 mM Tris buffer, pH 8.6. The unadsorbed, unreacted [ $^3\text{H}$ ] tyrosine was washed out of the column by the passage of 40 ml of 5 mM Tris buffer, pH 8.6.

### Elution of [ $^3\text{H}$ ] L-DOPA

The adsorbed [ $^3\text{H}$ ] L-DOPA on alumina was eluted from the column using 3 mls of 1 M glacial acetic acid (Analar).

### Counting

A sample of 1 ml of the 3 ml eluate was added to 10 ml of the aqueous scintillant NE-260. The radioactivity of tritium in the samples was estimated in scintillation counter, the number of counts over a 10 min period being recorded.

### Estimating the % recovery of [ $^3\text{H}$ ] L-DOPA by the method

The recovery of L-DOPA through the whole procedure was measured as follows:

10  $\mu\text{l}$  of [ $^3\text{H}$ ] L-DOPA (specific activity 2.5 Ci/mMol, Radiochemical Center, Amersham) was added, in duplicate, to tissue homogenate, containing no [ $^3\text{H}$ ] tyrosine, and then passed through the method. The estimated radioactivity was compared to that counted following the addition of similar amounts (10  $\mu\text{l}$ ) of [ $^3\text{H}$ ] L-DOPA directly to the effluent of duplicate tissue sample processed through the method. The percentage recovery

ranged between 49 - 52% in all the assays.

### Blanks

Tissue and reagent blanks were assayed, in duplicate, simultaneously with tissue samples. Tissue blanks, in duplicate, were prepared by heating the homogenate to 80°C for 15 min and then cooled on ice. 10 µl perchloric acid was then added before the addition of the tyrosine substrate solution. Reagent blanks, in duplicate, were prepared by mixing 10 µl of 20 mM KPO<sub>4</sub> buffer, pH 7.4 containing 1% of 0.1% Triton-X with 10 µl of the tyrosine substrate solution.

Tissue and reagent blanks gave similar low counts. Tissue blanks gave counts between 460 - 1000 counts/min and these were used during the calculation of the enzyme activity.

Nil values were assigned to all tissue samples with less than double the counts produced by tissue blanks.

### Calculations

$$\begin{array}{l} \text{TOH activity} \\ \text{(nMol } [^3\text{H}] \text{ L-DOPA} \\ \text{per hr per gm wet} \\ \text{tissue weight)} \end{array} = \frac{\text{DPM} \times \text{Recovery} \times 9 \times 1000}{49,333,000}$$

$$\begin{array}{l} \text{DPM of sample} \\ \text{(desintegrations/min)} \end{array} = \begin{array}{l} \text{(cpm)} \\ \text{Sample counts/min} \times \text{efficiency of counting} \\ \text{for Tritium.} \end{array}$$

$$\text{Sample cpm} = \text{Counted cpm minus cpm of tissue blank.}$$

In the assay, 1 µCi of [<sup>3</sup>H] tyrosine (equivalent to 2,220,000) was used in each reaction. This is equivalent to the activity present in 0.045 nMol tyrosine. Thus,

$$\begin{array}{l} 2,220,000 \text{ dpm} \\ 0.045 \text{ nMol L-DOPA} \end{array} = \begin{array}{l} 0.045 \text{ nMol tyrosine} \\ 0.045 \text{ nMol L-DOPA} \end{array}$$

$$\begin{array}{l} 1 \text{ nMol L-DOPA} \\ \end{array} = \frac{2,220,000}{0.045}$$

$$= 49,333,000 \text{ dpm.}$$

### Estimating the efficiency of counting for [ $^3\text{H}$ ]

Ten samples were used. In each sample, 5  $\mu\text{l}$  of [ $^3\text{H}$ ] toluene (standard activity 20,000 dpm/5  $\mu\text{l}$ ) was added to 10 ml of NE-260 scintillant and counted for 10 min. The estimated percentage efficiency for tritium ranged between 32-36%, with an average efficiency of 34%.

## 3. DOPAMINE AGONISTS AND KINDLING

### A. EFFECT OF SOME DOPAMINE AGONISTS ON RATE OF KINDLING

#### 1. Bromocryptine and the rate of kindling

Twenty one male Wistar rats were used in the experiments, of which 15 rats reached the final stage of convulsions. Eight of these rats were bromocryptine-treated and 7 rats were vehicle-treated controls.

The operative procedure for electrode implantation is similar to that described above. Post-operative care, parameters of amygdaloid stimulation as well as the staging of motor seizure are similar to those described above.

Stimulation of the left amygdala was done once daily, beginning on the 8th post-operative day. Bromocryptine was given daily in a dose of 20 mg/kg, (i.p.) at 9.30 a.m. Control rats received an equivalent volume of vehicle intraperitoneally. Stimulation was done 4 hours after the injection when the effect of bromocryptine on behaviour was marked. The rats appeared to have recovered from the effect of bromocryptine after 24 hrs of each injection.

In this experiment the number of amygdaloid stimulations  
of  
required to elicit the different stages/motor seizure was determined

for 8 bromocryptine-treated and 7 control rats. Furthermore, the duration of the evoked afterdischarge was recorded after each stimulation session (in secs) starting from the point of stimulation of each rat.

## 2. Apomorphine and the rate of kindling

Eighteen male Wistar rats were used in this experiment, of which 13 rats reached stage 5 seizure. Seven of these rats were apomorphine-treated and 6 were saline-injected controls. The operative procedure, post-operative care, stimulation parameters and staging of seizure were similar to those described before.

Daily stimulation was begun once daily on the 8th day. Apomorphine was given daily at 10.00 a.m. in a dose of 2 mg/kg i.p. Control rats received an equivalent volume of saline, i.p. Stimulation was done twenty minutes after the injection when the effect of apomorphine on behaviour was marked. Rats recovered from the drug effect after 1 hr of its administration. In this experiment also, the number of stimulations required to produce a generalized seizure in 7 apomorphine-treated and 6 saline-treated controls was determined. Moreover, the duration of elicited afterdischarge was measured for each rat.

### B. ACUTE EFFECT OF SOME DOPAMINE AGONISTS ON AFTERDISCHARGE THRESHOLD AND DURATION

These experiments were done on rats kindled during previous experiments. At this stage, the afterdischarge threshold is much

more stable than that seen in the early stages of kindling stimulation, and any alterations in the level of the threshold might be more easily detectable.

1. Effect of bromocryptine on afterdischarge threshold and duration

Seven of the kindled rats in the previous bromocryptine experiment were used for testing the afterdischarge threshold on 6 consecutive days. On the first day the threshold was measured in the non-drugged stage. On the 4th day the threshold was determined 4 hrs after bromocryptine (20 mg/kg i.p.) administration. On each of the following two days the threshold was tested in the non-drugged state. The afterdischarge duration (in secs) was measured after each stimulation.

The threshold was determined as follows: Each animal was stimulated several times with 15 min periods of rest between stimulations. The intensity of the stimulating current was varied while all other parameters of stimulation remained the same as in the previous experiment. The current intensity of the first stimulation train was 40  $\mu$ A. Stimulation intensity was increased by 20  $\mu$ A steps on each subsequent stimulation until an afterdischarge was elicited. The lowest current at which the afterdischarge was observed was considered to be the afterdischarge threshold. On the second day the same procedure was repeated to assess the stability of the threshold. On the 3rd day, all rats were given an initial stimulation of their individual afterdischarge threshold. The current intensity was then raised by 20  $\mu$ A steps if no afterdischarge was produced, or it was lowered by 20  $\mu$ A if one was seen.

This procedure was continued until afterdischarges were evoked for the ascending series, or were no longer observed in the descending series. Again, the lowest current at which they were observed was taken as the afterdischarge threshold.

On these 3 days, once the afterdischarge threshold was determined, the duration (in secs) of the ensuing afterdischarge was measured.

On the 4th day, rats received bromocryptine in a dose of 20 mg/kg intraperitoneally. The same procedure for determination of afterdischarge threshold as that on the 3rd day was followed 4 hrs after bromocryptine administration when its effect on behaviour was marked. The duration of the evoked afterdischarge was also measured. The same procedure for determination of afterdischarge threshold and duration was followed on the next 2 days to detect any recovery or change in the threshold.

## 2. Effect of apomorphine on afterdischarge threshold and duration

Seven experimental rats that were kindled from the previous apomorphine experiment were used for testing the afterdischarge threshold and duration on 5 consecutive days. The parameters of stimulation and the design of the experiment was the same as that described with bromocryptine, except that the rats received apomorphine in a dose of 2 mg/kg i.p. on the 4th day and stimulation was done 20 min after the injection when the effects of apomorphine on behaviour were marked.

3. Effect of L-DOPA pretreatment on the afterdischarge threshold and duration

Six experimental rats kindled from previous experiments were used for testing the afterdischarge threshold and duration on 5 consecutive days. Essentially, the design of the experiment is the same as that described for apomorphine. However, L-DOPA was given in a dose of 250 mg/kg i.p. on the 4th day. When the behavioural effects with this dose, i.e. bristling of fur and increased locomotion, were seen after 20 min, stimulation of the amygdala was commenced to determine the threshold and duration of the evoked afterdischarge.



## RESULTS

### 1. AFTERDISCHARGE THRESHOLD AND KINDLING

The rats developed afterdischarge on the first day of stimulation. The threshold current for afterdischarge showed a progressive reduction with the progression of kindling stimulations. During the early stages the threshold was variable between different rats, and in the same rat at different occasions. However, as kindling progressed, the threshold became more stabilized with the development of different stages of the seizure, and was quite stable when generalized convulsion occurred. Fig. 30A shows the evolution of the afterdischarge threshold in each of 6 rats in the experiment. Generalized convulsions (stage 5) occurred after 8 to 12 stimulations.

Stage 1 seizure occurred after an average of 2.5 stimulations ( $n = 6$ ), the mean afterdischarge threshold at this stage was  $140 \mu\text{A} \pm 22 \text{ S.E.M.}$  (see Fig. 30B).

Stage 3 seizure occurred after an average of 6 stimulations ( $n = 6$ ) the mean threshold for afterdischarge at this stage was  $86 \mu\text{A} \pm 16 \text{ S.E.M.}$  (see Fig. 30B).

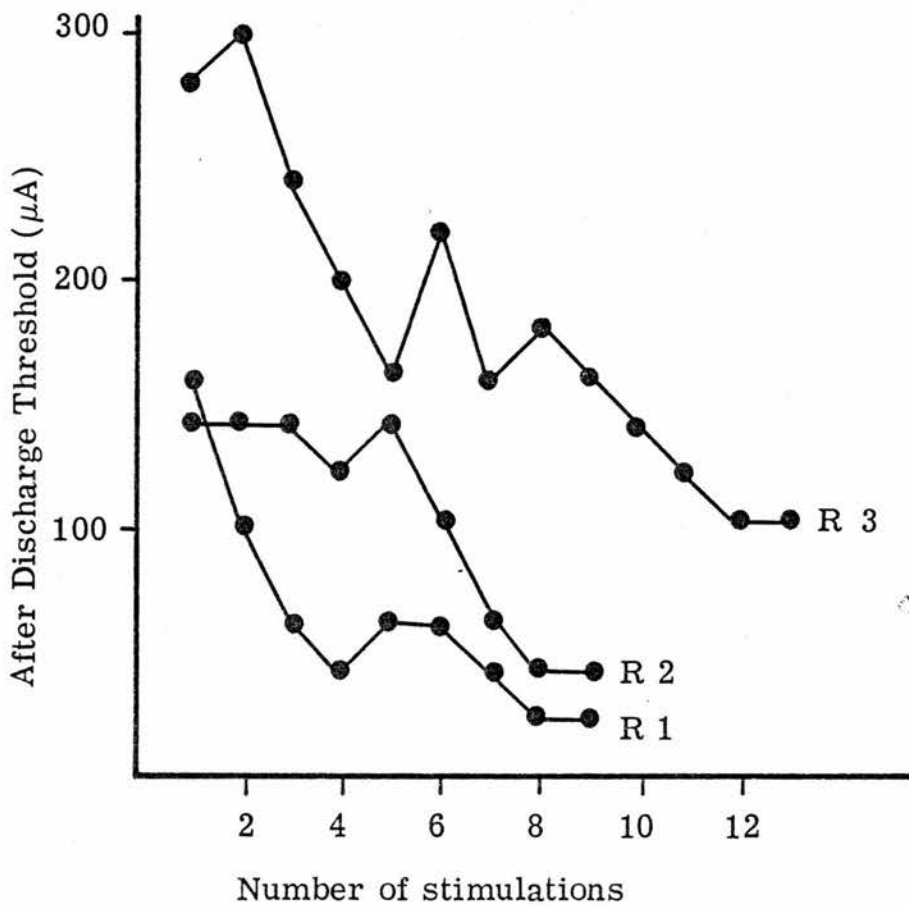
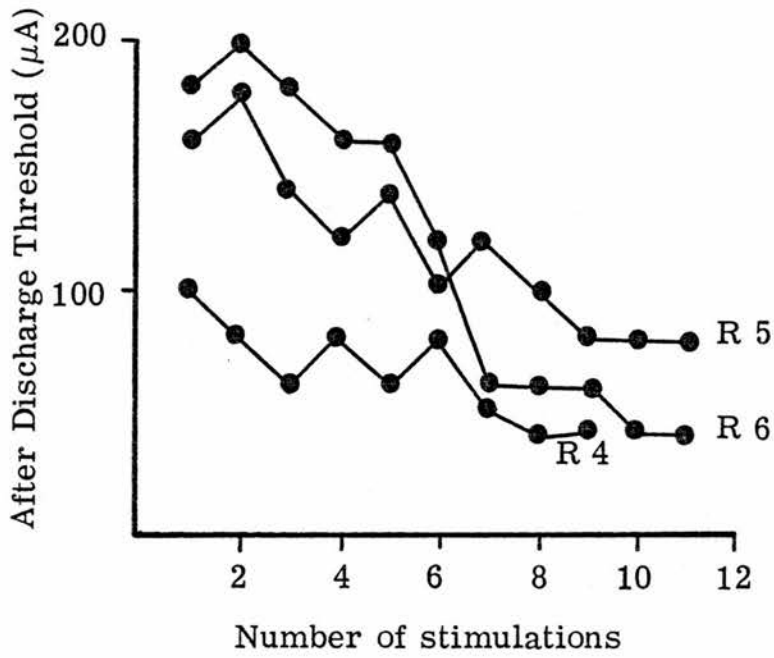
Stage 5 seizure occurred after an average of 9.5 stimulations ( $n = 6$ ) the average threshold for afterdischarge at this stage was  $55 \mu\text{A} \pm 10 \text{ S.E.M.}$  (see Fig. 30B).

### 2. RESULTS OF TYROSINE HYDROXYLASE IN KINDLING

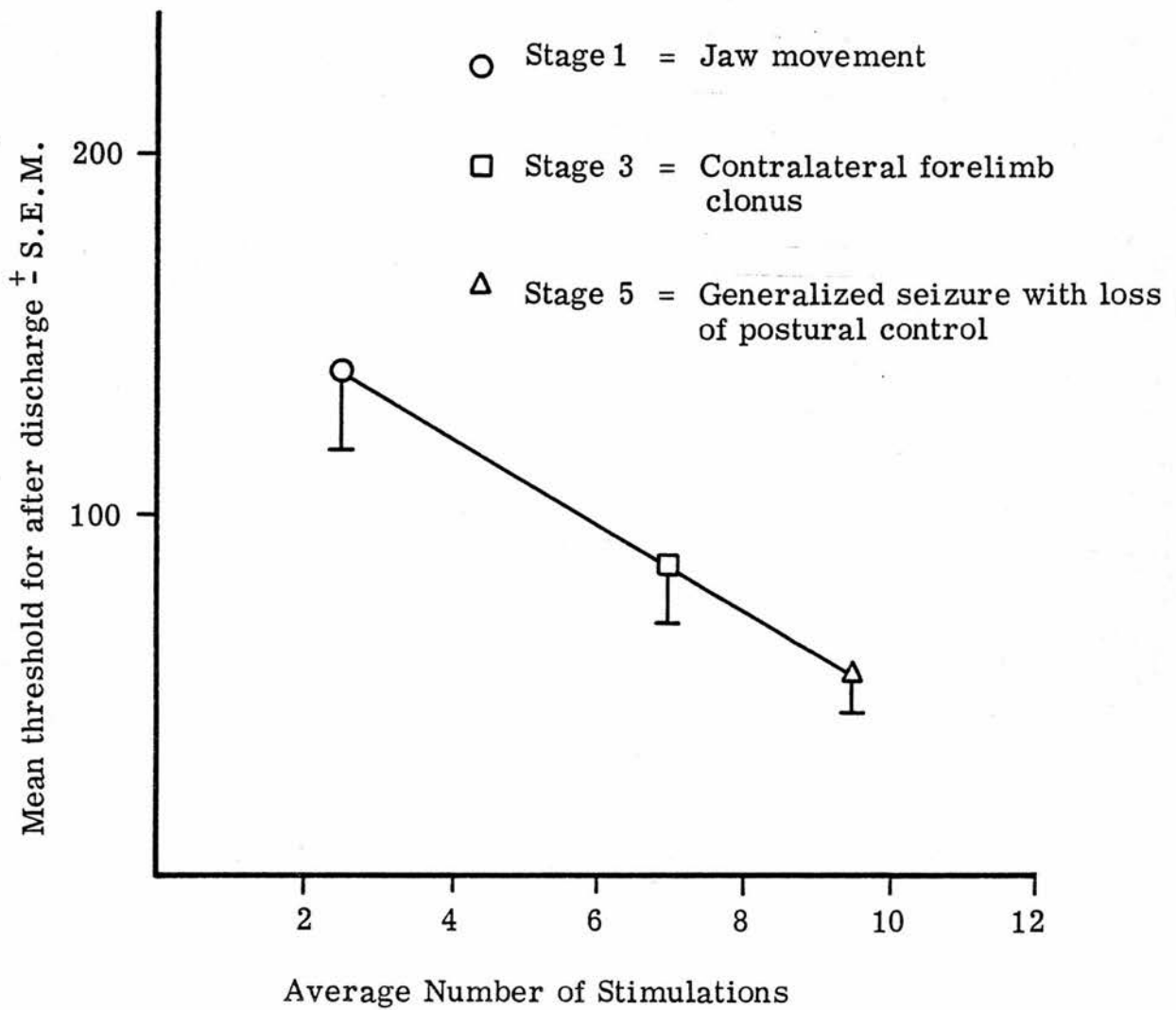
#### A. ANIMAL EXPERIMENT

All rats developed afterdischarges on the first day of stimulation

Showing the changes in after discharge threshold during the development of kindling in 6 rats.

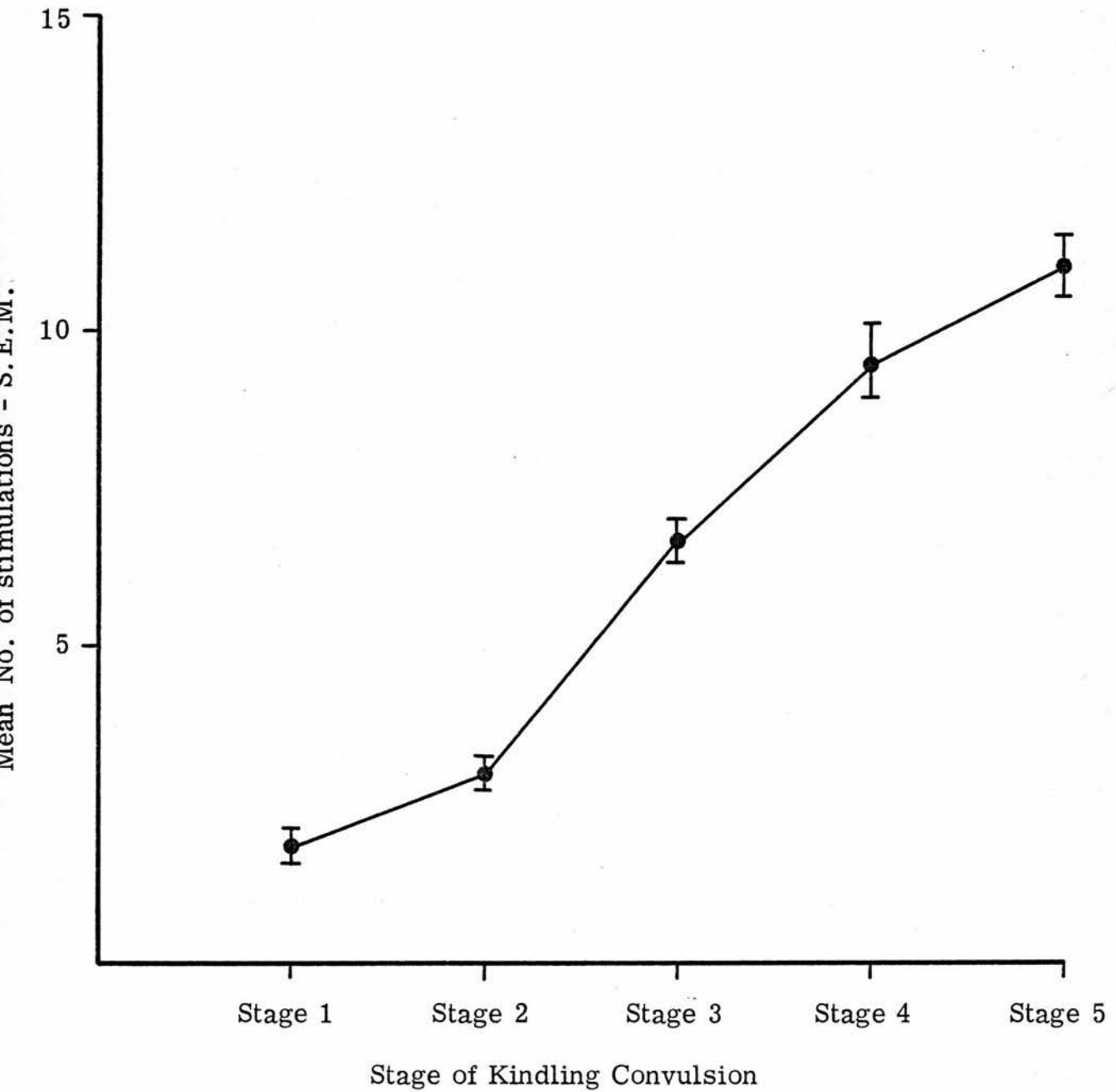


The after discharge threshold during different stages of kindling.



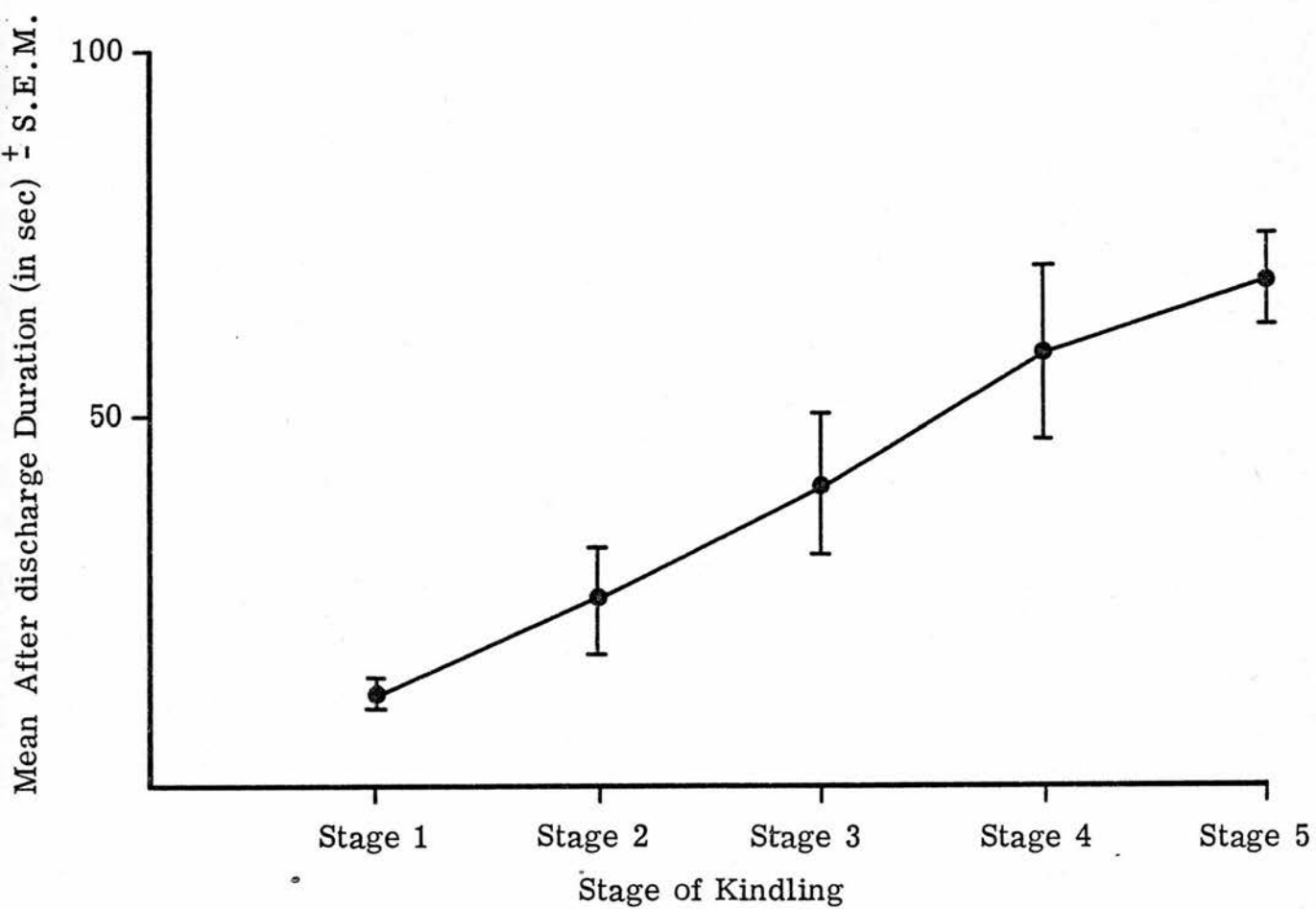
Number of stimulations required for different stages of seizure  
development in kindling       $n = 7$

- Rats were subsequently used for tyrosine hydroylase assay



Duration of after discharge (in sec) during the different stages  
of seizure development in kindling       $n = 7$

- Rats were subsequently used for tyrosine hydroxylase assay



of the amygdala. The early stages of motor seizures were seen after few stimulations in most rats. In 2 rats, however, mouth twitches and head nodding were evident on the first stimulation. All rats developed generalized convulsions after 9 to 13 days of daily amygdaloid stimulations. The pattern and rate of seizure developed and the spread of afterdischarge was similar to that seen with kindling induced by stimulation of the amygdala. Figures 31A and 31B show the average number of stimulations required for the development of different stages of kindling convulsions in this group of rats, and the mean duration of afterdischarge at these stages, respectively.

#### B. BIOCHEMICAL ANALYSIS

Tyrosine hydroxylase in brain area of unoperated control rats was not significantly different from the normal levels reported by other workers (Uretsky & Iversen, 1970). The results of tyrosine hydroxylase activity in the amygdala of the 3 groups of rats studied are shown in Table 18 and the Summary table. Analysis of variance showed no significant difference in the enzyme activity in the right and left amygdalae of both sham-operated and unoperated control rats. The data, however, showed a persistent and significant reduction in enzyme activity in the left stimulated, but not the contralateral amygdala of all kindled rats in comparison to controls (Two-way Analysis of variance, F-distribution,  $P = 0.05$ ). This reduction in enzyme activity was significant at the level of  $P < 0.002$  in comparison (Mann-Whitney D test, two-tailed,  $u = 3$ ) to that of the contralateral side. However, no correlation was found between the reduced enzyme

SUMMARY TABLE

TYROSINE HYDROXYLASE ACTIVITY (nMol  $^3\text{H}$ -L-DOPA formed/hr/g wet weight)  
IN DIFFERENT BRAIN REGIONS IN AMYGDALOID KINDLED RATS

- each value represents the mean activity  $\pm$  S.E. (n)

	Right	Left
<u>Amygdala</u>		
Kindled (7)	1.96 $\pm$ 0.13	#1.31 $\pm$ 0.09*
Sham-operated (7)	2.11 $\pm$ 0.17	#2.10 $\pm$ 0.15
Unoperated (7)	1.95 $\pm$ 0.10	1.98 $\pm$ 0.07
<u>Hippocampus</u>		
Kindled (6)	0.36 $\pm$ 0.02	0.34 $\pm$ 0.04
Sham-operated (6)	0.38 $\pm$ 0.03	0.42 $\pm$ 0.02
Unoperated (6)	0.38 $\pm$ 0.02	0.40 $\pm$ 0.03
<u>Hypothalamus</u>		
Kindled (6)	4.17 $\pm$ 0.28	3.58 $\pm$ 0.28
Sham-operated (6)	4.31 $\pm$ 0.19	4.45 $\pm$ 0.25
Unoperated (6)	5.01 $\pm$ 0.18	4.89 $\pm$ 0.27
<u>Thalamus</u>		
Kindled (6)	1.13 $\pm$ 0.11	0.82 $\pm$ 0.16
Sham-operated (6)	0.89 $\pm$ 0.10	0.88 $\pm$ 0.10
Unoperated (6)	1.10 $\pm$ 0.18	0.92 $\pm$ 0.16
<u>Striatum</u>		
Kindled (6)	23.99 $\pm$ 1.05	21.79 $\pm$ 0.68
Sham-operated (6)	25.02 $\pm$ 1.63	24.62 $\pm$ 1.24
Unoperated (6)	23.99 $\pm$ 1.66	25.14 $\pm$ 1.85
<u>Cortex</u>		
Kindled (7)	0.28 $\pm$ 0.01	0.26 $\pm$ 0.02
Sham-operated (7)	0.27 $\pm$ 0.02	0.27 $\pm$ 0.02
Unoperated (7)	0.27 $\pm$ 0.02	0.28 $\pm$ 0.02

# Site of electrode implantation into the left amygdala

\*  $P < 0.002$  for comparison of right and left sides (Mann-Whitney-U-test)

TABLE 18

TYROSINE HYDROXYLASE ACTIVITY (nMol  $^3\text{H}$ -L-DOPA/hr/g wet tissue weight)  
IN THE AMYGDALA OF KINDLED RATS

Sample	Kindled Rats		Sham-operated Controls		Unoperated Controls	
	Right	Left*	Right	Left*	Right	Left
1	2.04	1.58	2.33	2.31	1.85	1.92
2	2.23	1.58	1.83	2.07	1.90	1.94
3	1.65	1.07	1.46	1.66	1.74	1.95
4	1.63	1.03	2.53	2.13	2.31	2.15
5	2.36	1.56	2.43	2.22	2.22	2.16
6	2.38	1.40	2.66	2.82	2.13	2.12
7	1.44	0.97	1.57	1.48	1.51	1.60
Mean	1.96	1.31#	2.11	2.10	1.95	1.98
± S.E.M.	0.13	0.09	0.17	0.15	0.10	0.07

Two-way analysis of variance showed the enzyme activity to be significantly reduced in the left stimulated amygdala of kindled rats (F-distribution,  $P = 0.05$ ) in comparison to that of the right amygdala of the 3 groups of rats.

\* Site of implanted electrode (left amygdala)

#  $P < 0.002$  (Mann-Whitney,  $U = 3$ ) for comparison of right and left sides



TABLE 19

TYROSINE HYDROXYLASE ACTIVITY (nMol  $^3\text{H}$ -L-DOPA/hr/g wet tissue weight)  
IN THE HIPPOCAMPUS OF KINDLED RATS

Sample	Kindled Rats		Sham-operated Controls		Unoperated Controls	
	Right	Left	Right	Left	Right	Left
1	0.02	0.29	0.38	0.42	0.46	0.42
2	0.35	0.28	0.40	0.38	0.38	0.44
3	0.34	0.54	0.51	0.52	0.34	0.52
4	0.34	0.36	0.34	0.42	0.47	0.46
5	0.47	0.30	0.31	0.37	0.32	0.39
6	0.32	0.31	0.39	0.47	0.38	0.41
7	-	-	-	-	-	-
Mean	0.36	0.34	0.38	0.42	0.38	0.40
+ S.E.M.	0.02	0.03	0.03	0.02	0.02	0.03

The differences in enzyme activity between the three groups of rats were not statistically significant (Analysis of Variance)

(-) sample lost during the assay

TABLE 20

TYROSINE HYDROXYLASE ACTIVITY (nMol  $^3\text{H}$ -L-DOPA/hr/g wet tissue weight)  
IN THE HYPOTHALAMUS OF KINDLED RATS

Sample	Kindled Rats		Sham-operated Controls		Unoperated Controls	
	Right	Left	Right	Left	Right	Left
1	3.94	3.43	3.71	3.72	4.42	3.66
2	2.99	3.69	3.74	4.11	5.35	5.12
3	3.80	2.63	4.43	3.83	4.70	4.82
4	4.97	4.52	4.54	4.81	4.96	5.59
5	4.94	2.96	5.05	5.48	5.75	5.56
6	4.36	4.30	4.37	4.76	4.86	4.46
7	-	-	-	-	-	-
Mean	4.17	3.58	4.31	4.45	5.01	4.89
+ S.E.M.	0.28	0.28	0.19	0.25	9.18	0.27

The differences in enzyme activity between the three groups of rats were not statistically significant

(-) = sample lost during the assay

TABLE 21

## TYROSINE HYDROXYLASE ACTIVITY IN STRIATUM OF KINDLED RATS

- enzyme activity in nMol  $^3\text{H}$ -L-DOPA formed/hr/g wet tissue weight
- S.E. = standard error of the mean

Number of tissue sample	Kindled Rats		Sham-operated Controls		Unoperated Congrols	
	Right	Left	Right	Left	Right	Left
1	24.85	20.86	27.74	27.53	26.00	30.68
2	25.92	23.65	29.31	28.26	20.48	23.93
3	22.61	20.12	22.93	23.02	19.34	21.60
4	23.45	24.52	20.33	19.29	21.40	18.91
5	27.58	20.74	25.32	25.99	25.50	24.38
6	19.53	20.89	24.48	23.61	31.23	31.34
7	-	-	-	-	-	-
Mean	23.99	21.79	25.02	24.62	23.99	25.14
+ S.E.	1.05	6.68	1.63	1.24	1.66	1.84

The differences in enzyme activity between the three groups of rats were not statistically significant (Two-way analysis of variance, F-distribution)

(-) = sample lost during the assay

TABLE 22

TYROSINE HYDROXYLASE ACTIVITY (nMol  $^3\text{H}$ -L-DOPA/hr/g wet tissue weight)  
IN THE FRONTAL CORTEX OF KINDLED RATS

Sample	Kindled Rats		Sham-operated Controls		Unoperated Controls	
	Right	Left	Right	Left	Right	Left
1	0.28	0.22	0.23	0.27	0.26	0.23
2	0.27	0.28	0.31	0.35	0.36	0.41
3	0.37	0.33	0.23	0.22	0.32	0.30
4	0.29	0.20	0.36	0.33	0.36	0.31
5	0.28	0.34	0.26	0.25	0.26	0.27
6	0.21	0.23	0.23	0.25	0.28	0.27
7	0.24	0.25	0.24	0.22	0.20	0.22
Mean	0.28	0.26	0.27	0.27	0.29	0.28
+ S.E.M.	0.01	0.02	0.02	0.02	0.02	0.02

The differences in enzyme activity between the three groups of rats were not significant (Analysis of Variance)

TABLE 23

TYROSINE HYDROXYLASE ACTIVITY (nMol  $^3\text{H}$ -L-DOPA/hr/g wet tissue weight)  
IN THE THALAMUS OF KINDLED RATS

Sample	Kindled Rats		Sham-operated Controls		Unoperated Controls	
	Right	Left	Right	Left	Right	Left
1	1.54	1.63	1.07	1.12	1.34	0.87
2	1.21	0.82	0.73	0.56	1.86	1.61
3	0.76	0.70	0.67	0.58	0.79	0.67
4	0.88	0.75	0.60	0.83	1.29	1.20
5	1.40	0.61	1.25	1.21	0.54	0.48
6	0.99	0.41	1.01	0.99	0.78	0.69
7	-	-	-	-	-	-
Mean	1.13	0.82	0.89	0.88	1.10	0.92
$\pm$ S.E.M.	0.11	0.16	0.10	0.10	0.18	0.16

The differences in enzyme activity between the three groups of rats were not statistically significant

(-) = sample lost during the assay

activity and the pattern or rate of seizure development or the duration of the evoked afterdischarge.

No significant differences was found in tyrosine hydroxylase activity in the hippocampus, thalamus, hypothalamus, striatum and frontal cortex on the right and left sides of both implanted and unoperated control rats (see Tables 19-23 and Summary table).

In kindled rats, no significant difference was found in the enzyme activity in the hippocampus thalamus and frontal cortex on both sides of the brain. However, although the enzyme activity tended to be reduced in the striatum and hypothalamus, this reduction was not statistically significant in kindled rats.

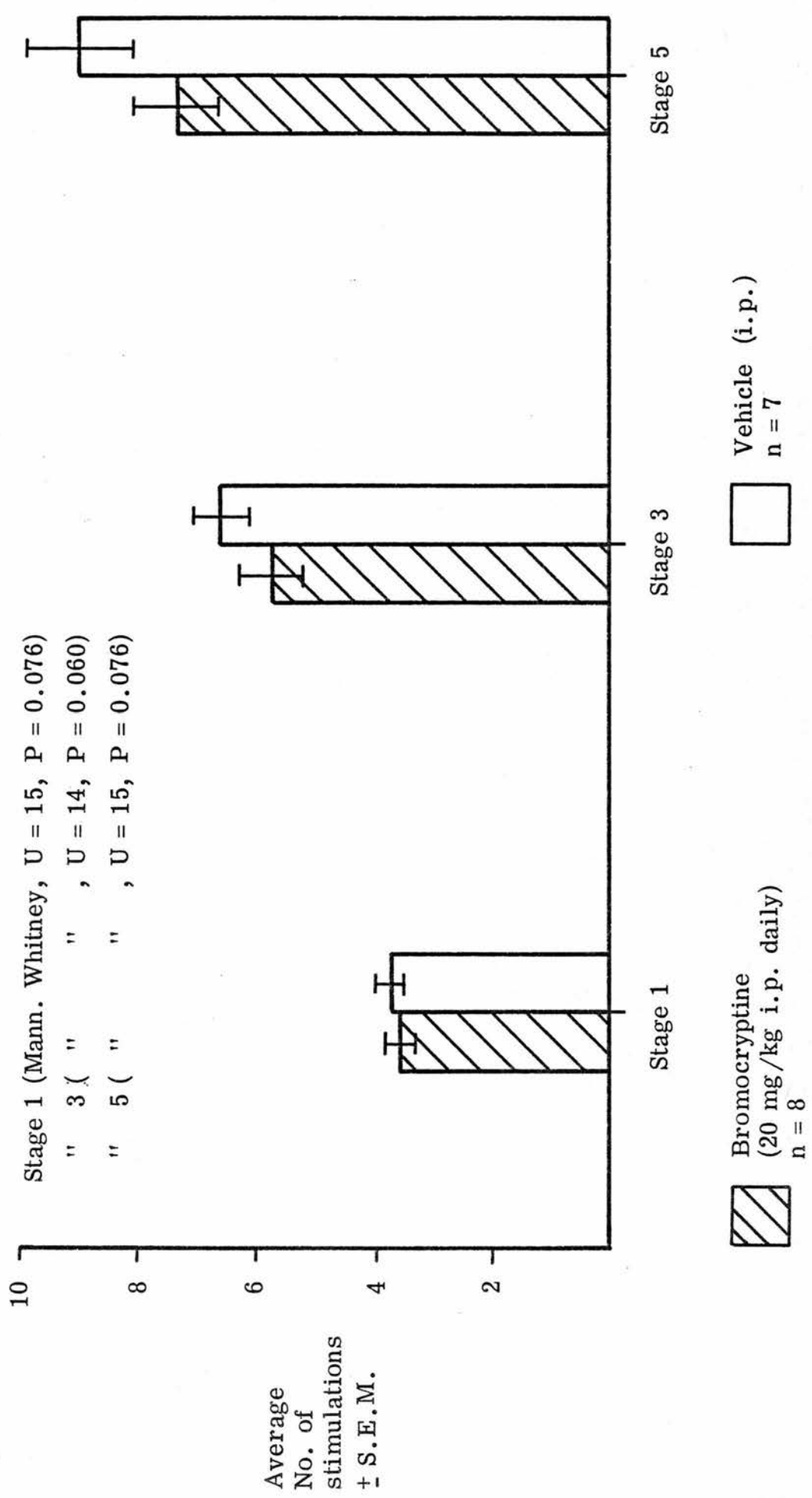
### 3. EFFECT OF DOPAMINE AGONISTS ON KINDLING

#### A. EFFECT ON THE RATE OF KINDLING

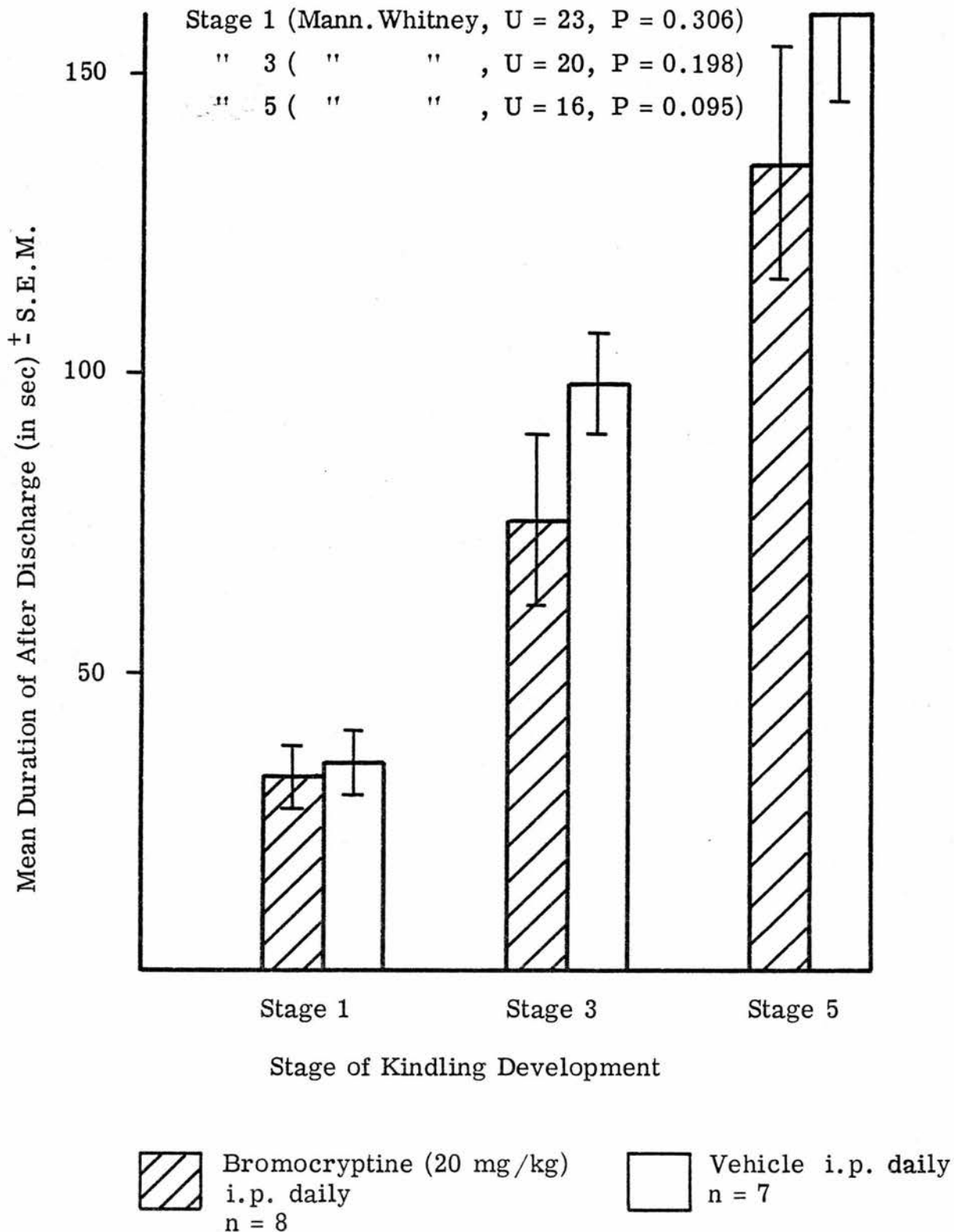
(Figs. 32A, B & C, Figs. 33A, B & C). The onset of motor seizure was not significantly different in drug-treated and control rats (Mann-Whitney-U-test). The mean number of stimulations required to elicit stage 1 seizures was 3.6 ( $n = 8$ ) for bromocryptine-treated and 2.7 ( $n = 7$ ) for the apomorphine-treated in comparison to 3.7 ( $n = 7$ ) for vehicle-treated, and 2.6 ( $n = 6$ ) for saline-injected controls, respectively. Similarly, the average duration of after-discharge at stage 1 was 32.6 ( $n = 8$ ) sec for bromocryptine-treated and 47.8 ( $n = 7$ ) sec for apomorphine-treated, in comparison to 35 sec ( $n = 7$ ) for vehicle-treated and 43.4 ( $n = 6$ ) sec for saline controls, respectively. The differences were not statistically significant (Mann-Whitney-U-test).

The development of stage 3 seizure, i.e. contralateral forelimb

Effect of chronic administration of bromocryptine (20 mg/kg i.p. daily) on the number of stimulations required to produce the different stages of seizures induced in rats by daily amygdaloid stimulation.

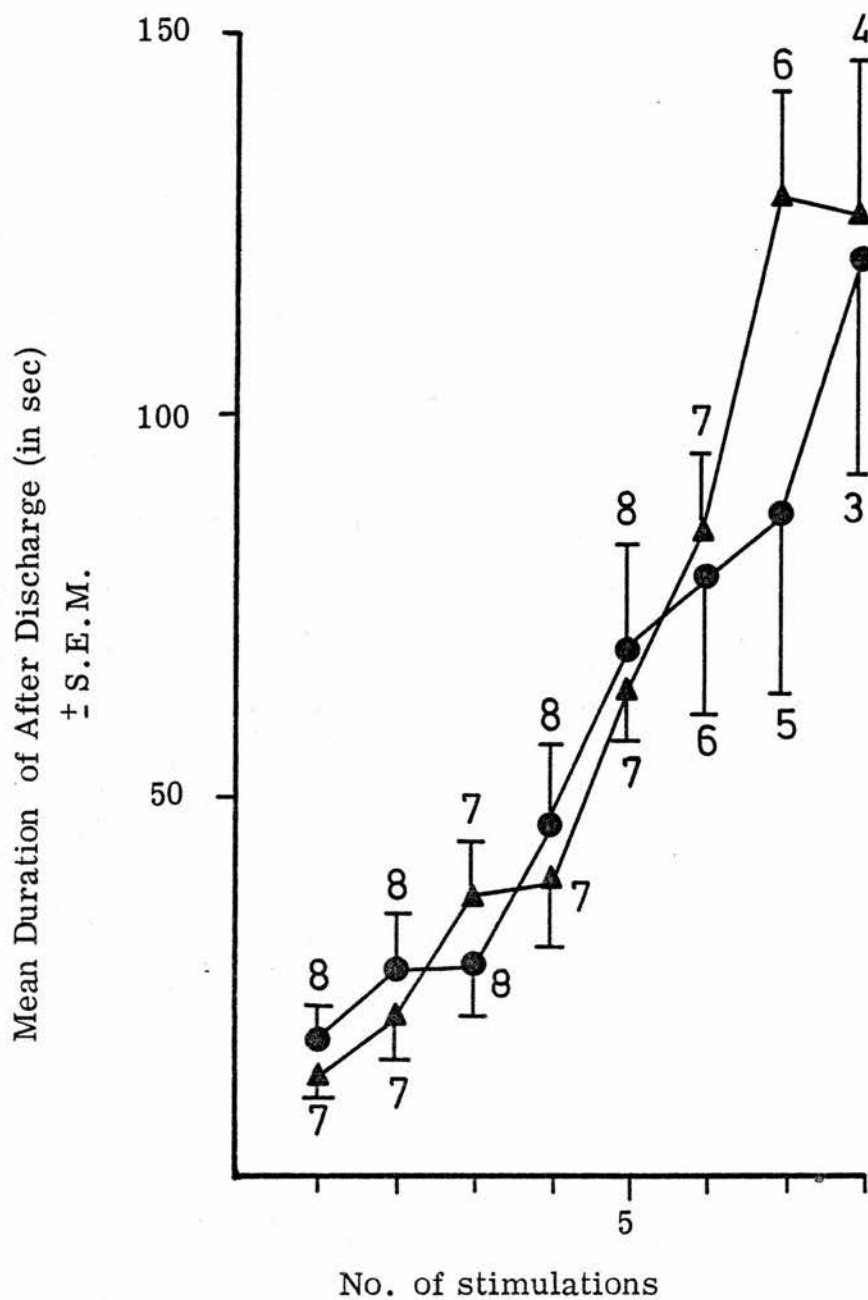


Effect of chronic administration of bromocryptine (20 mg/kg i.p.) on the duration of after discharge (in sec) during different stages of seizure development in rats kindled by daily amygdaloid stimulation.





Rate of increase in after discharge duration following bromocryptine (20 mg/kg i.p. daily) administration in rats kindled by daily amygdaloid stimulation.



●—● Bromocryptine (n)

▲—▲ Controls (n)

Day 2 (Mann. Whitney,  $U = 22$ ,  $p = 0.347$ )

" 4 ( " " ,  $U = 25$ ,  $p = 0.389$ )

" 6 ( " " ,  $U = 18$ ,  $p = 0.365$ )

" 8 ( " " ,  $U = 5$ ,  $p = 0.429$ )

clonus, was not significantly different in drug-treated and control rats (Mann-Whitney-U-test). The average number of stimulations required to elicit this stage of seizure was 5.75 ( $n = 8$ ) for bromocryptine-treated and 5.86 ( $n = 7$ ) for apomorphine-treated, in comparison to 6.6 ( $n = 7$ ) for vehicle-treated and 6.33 ( $n = 6$ ) for saline controls, respectively. The average duration of afterdischarge at this stage was 75.6 ( $n = 8$ ) sec for bromocryptine-treated and 77.4 ( $n = 7$ ) sec for apomorphine-treated in comparison to 98.3 ( $n = 7$ ) sec for vehicle-treated and 66 ( $n = 6$ ) sec for saline-injected controls respectively. The differences were not statistically significant (Mann-Whitney-U-test).

Moreover, the average number of stimulations required to elicit generalized convulsions (stage 5) was 7.4 ( $n = 8$ ) for bromocryptine-treated and 9.86 ( $n = 7$ ) for apomorphine-treated in comparison to 9 ( $n = 7$ ) for vehicle-treated and 9.83 ( $n = 6$ ) sec for saline controls, respectively. The differences were not statistically significant (Mann-Whitney-U-test). The average duration of afterdischarge at this stage was 135 sec ( $n = 8$ ) for bromocryptine-treated and 117 sec ( $n = 7$ ) for apomorphine-treated, in comparison to 160 sec ( $n = 7$ ) in vehicle-treated and 134 sec ( $n = 6$ ) in saline-treated control rats, respectively. The differences were not statistically significant (Mann-Whitney-U-test).

It might be worth to mention that in spite of increment in the afterdischarge duration, it remained variable between rats and in the same rat as the final stages of kindling convulsions were approached. This is in contrast to the more stable lowered afterdischarge threshold at that period, as already seen in the previous experimentation

The effect of chronic administration of apomorphine (2 mg/kg i.p. daily) on the rate of development of different stages of seizures in rats kindled by daily amygdaloid stimulation.

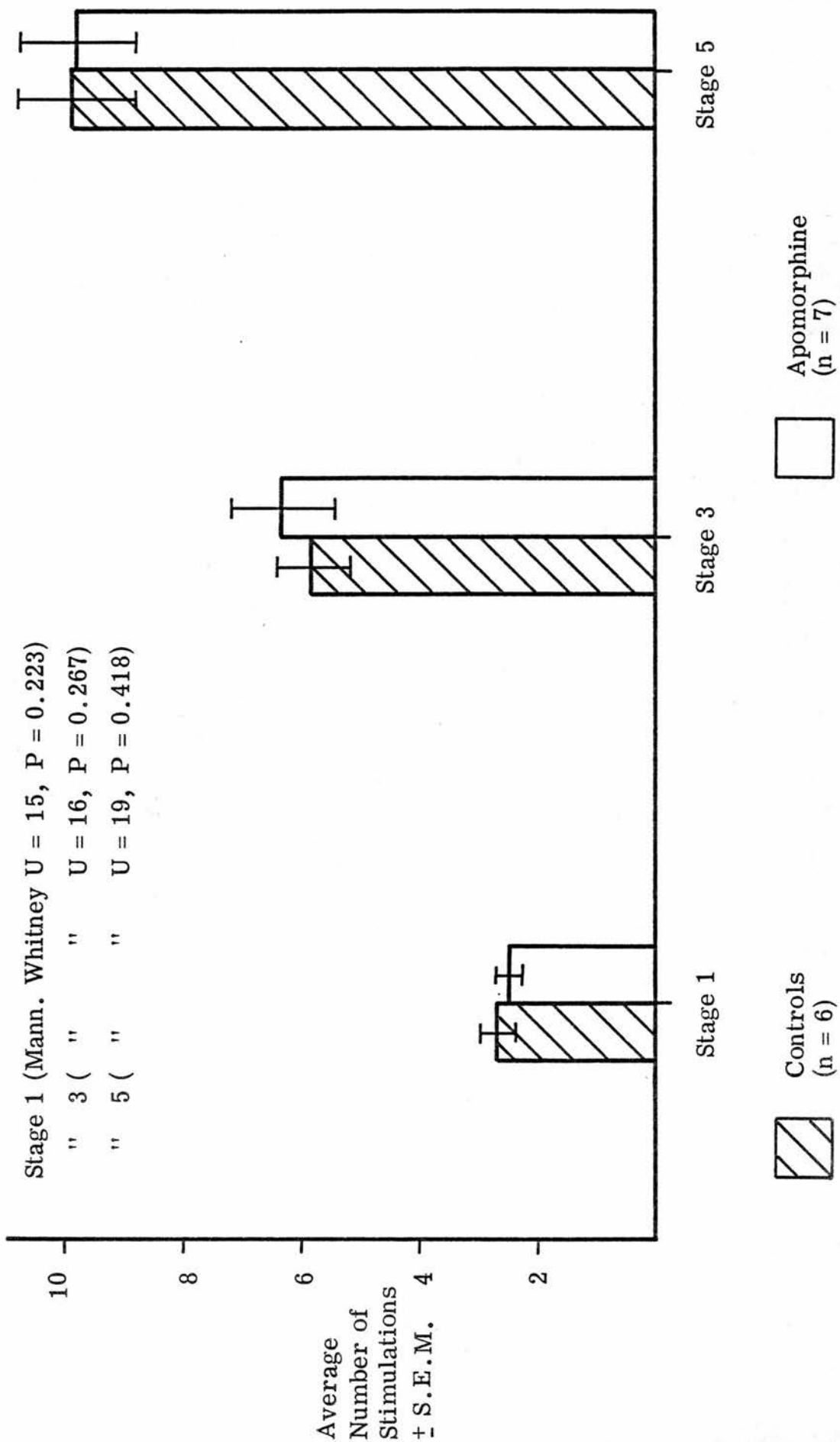
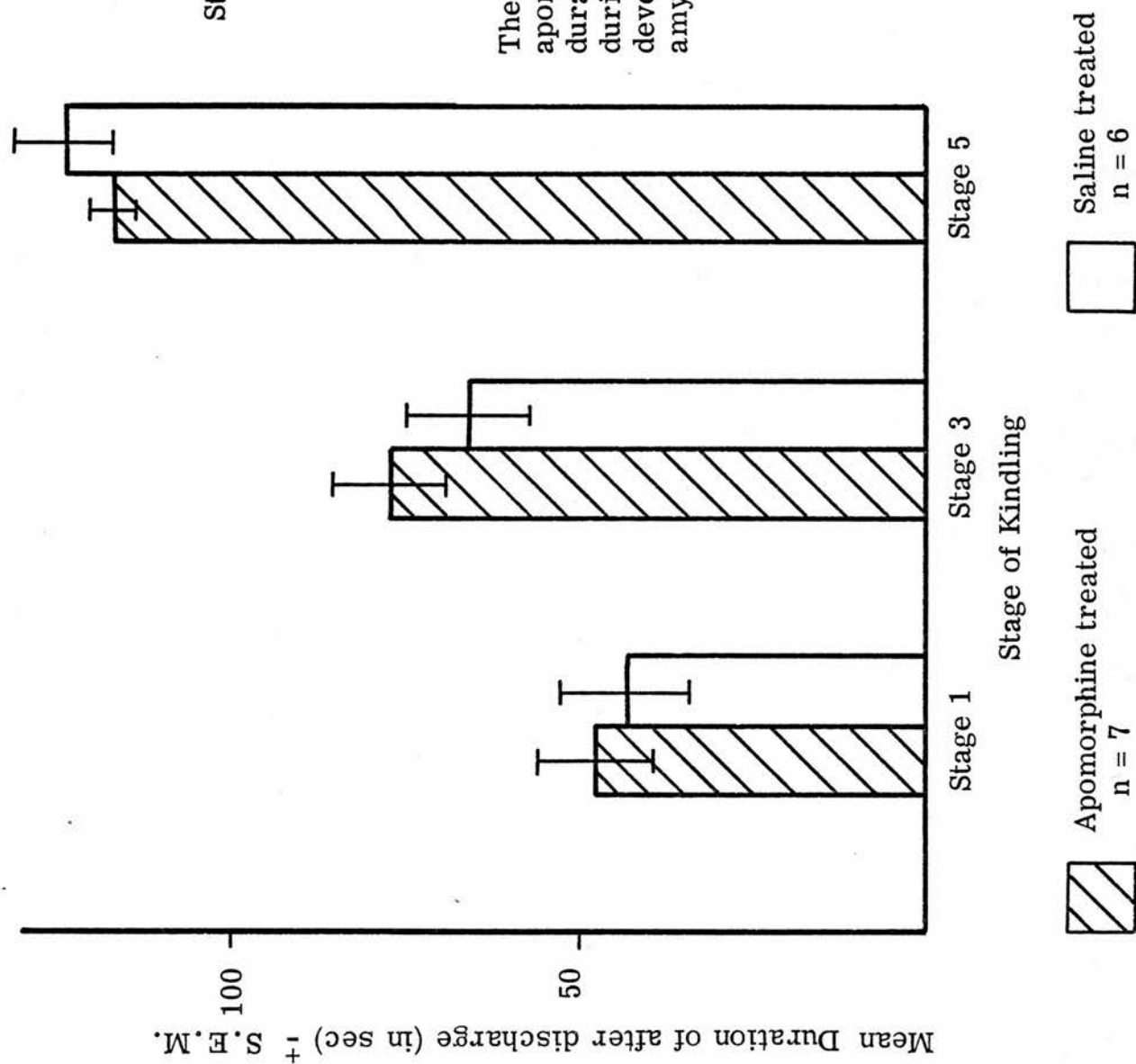


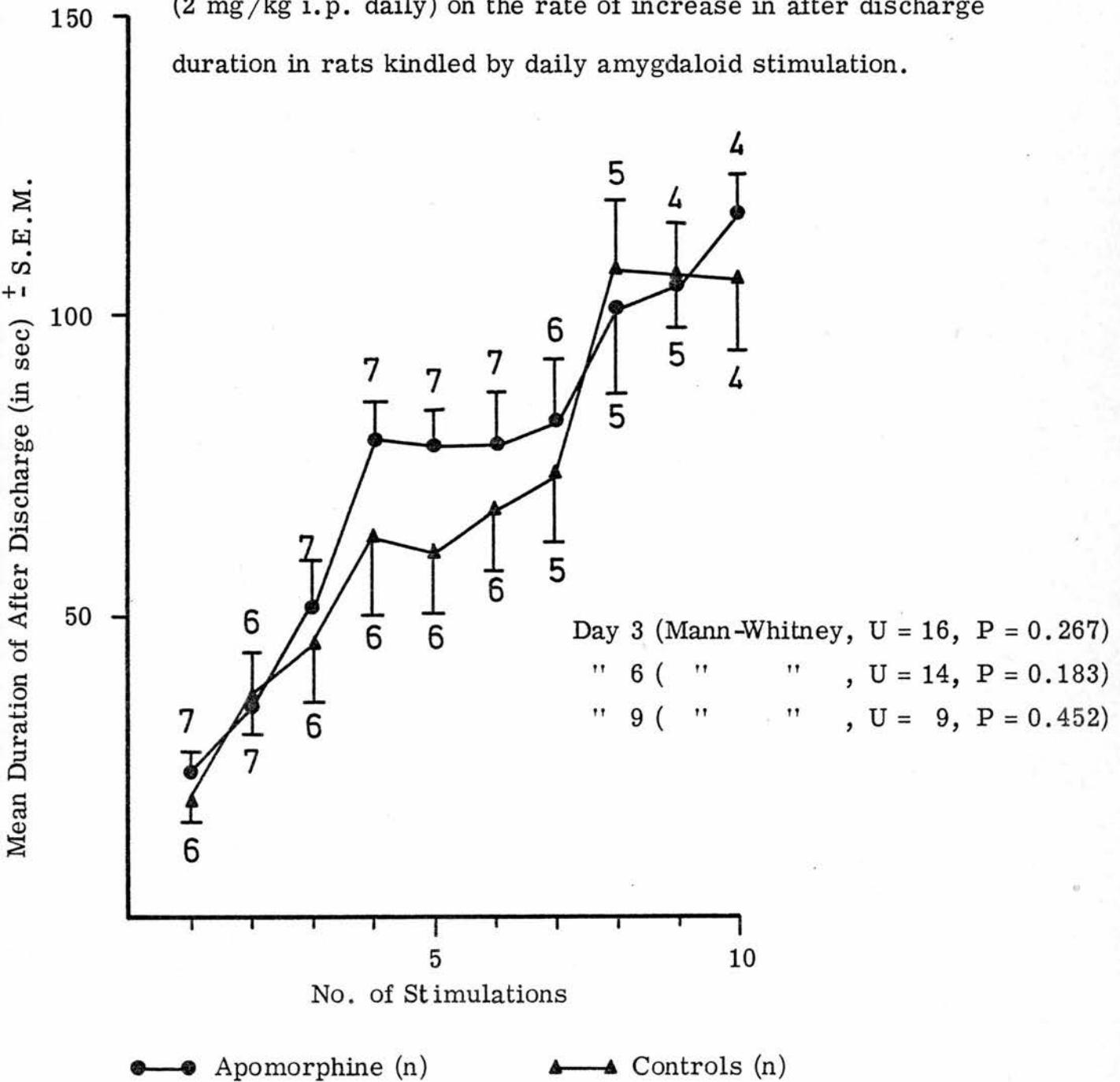
Fig 33B



Stage 1 (Mann. Whitney,  $U = 18$ ,  $P = 0.365$ )  
 " 3 ( " " ,  $U = 17$ ,  $P = 0.314$ )  
 " 5 ( " " ,  $U = 17$ ,  $P = 0.314$ )

The effect of daily administration of apomorphine (2 mg/kg i.p.) on the duration of after discharge (in sec) during the different stages of seizure development in rats kindled by daily amygdaloid stimulation

The effect of chronic administration of apomorphine  
(2 mg/kg i.p. daily) on the rate of increase in after discharge  
duration in rats kindled by daily amygdaloid stimulation.



on the afterdischarge threshold.

Moreover, there was no significant difference in the rate of increase in afterdischarge duration in bromocryptine and apomorphine-treated rats in comparison to their respective controls. When compared after an equal number of stimulations, the duration of afterdischarge was not statistically different between the drug-treated and control rats (Mann-Whitney-U-test).

Brain tissue sections (30 microns each) showed that the electrode tracts led to the region of the basolateral amygdala. This was confirmed in some rats by histological examination of stained sections. Besides, the pattern of seizure development strongly indicates that the site of electrode implantation was the amygdala.

B. EFFECT OF DOPAMINE AGONISTS ON AFTERDISCHARGE THRESHOLD AND DURATION

1. EFFECT ON AFTERDISCHARGE THRESHOLD

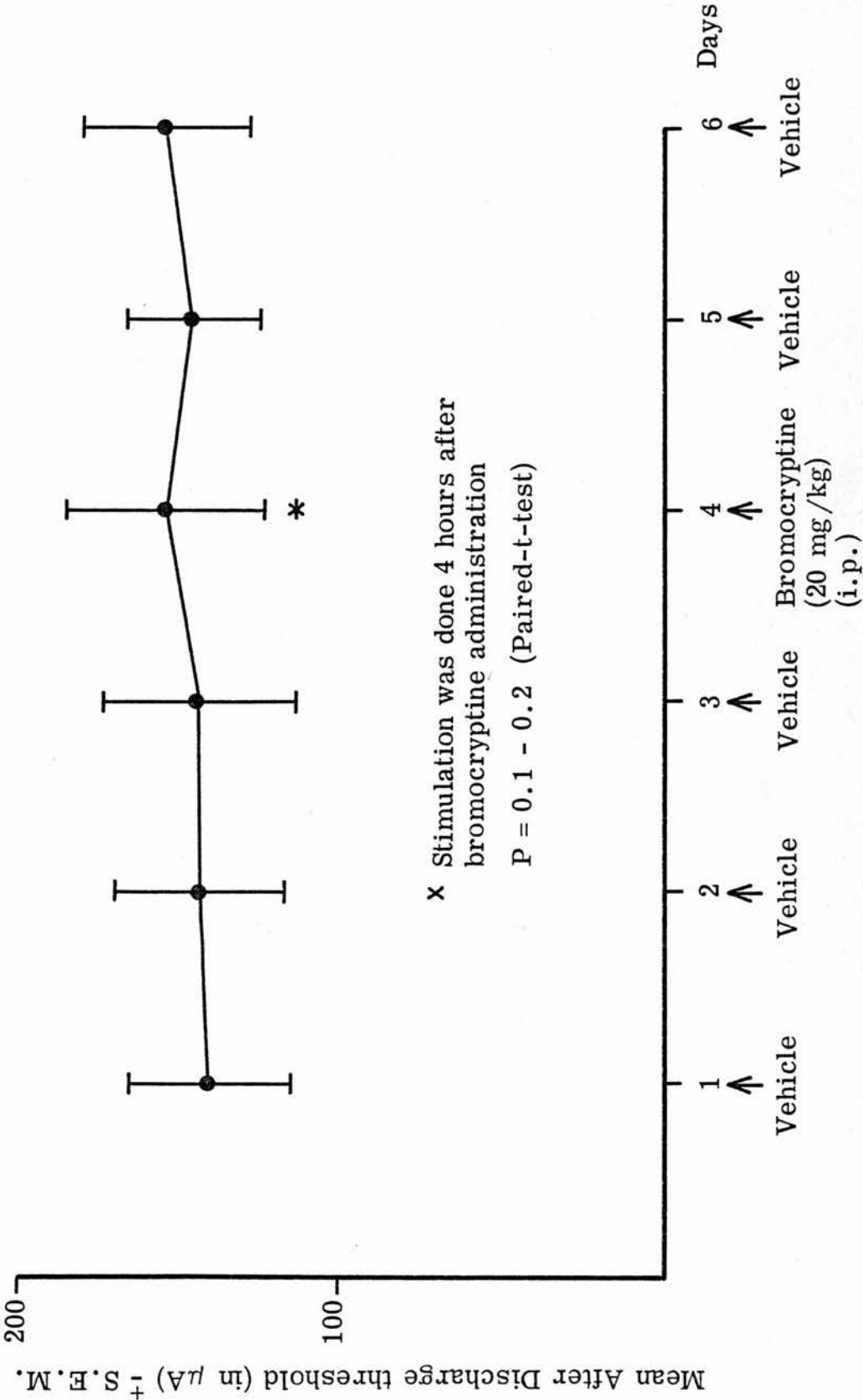
The afterdischarge threshold was measured on each of 3 consecutive days and were found to be stable. In the 7 kindled rats of the bromocryptine experiments, they ranged between 60 to 260  $\mu$ A with a mean threshold of 142  $\mu$ A. In the 7 kindled rats in the apomorphine experiments, the afterdischarge threshold ranged between 160 to 280  $\mu$ A with a mean threshold of 203  $\mu$ A, while in the 6 kindled rats of the L-DOPA experiments, they ranged between 100 to 240  $\mu$ A with a mean threshold of 170  $\mu$ A.

On the second or third day some rats showed a 20 or 40  $\mu$ A increase or decrease while others showed no change in the level of

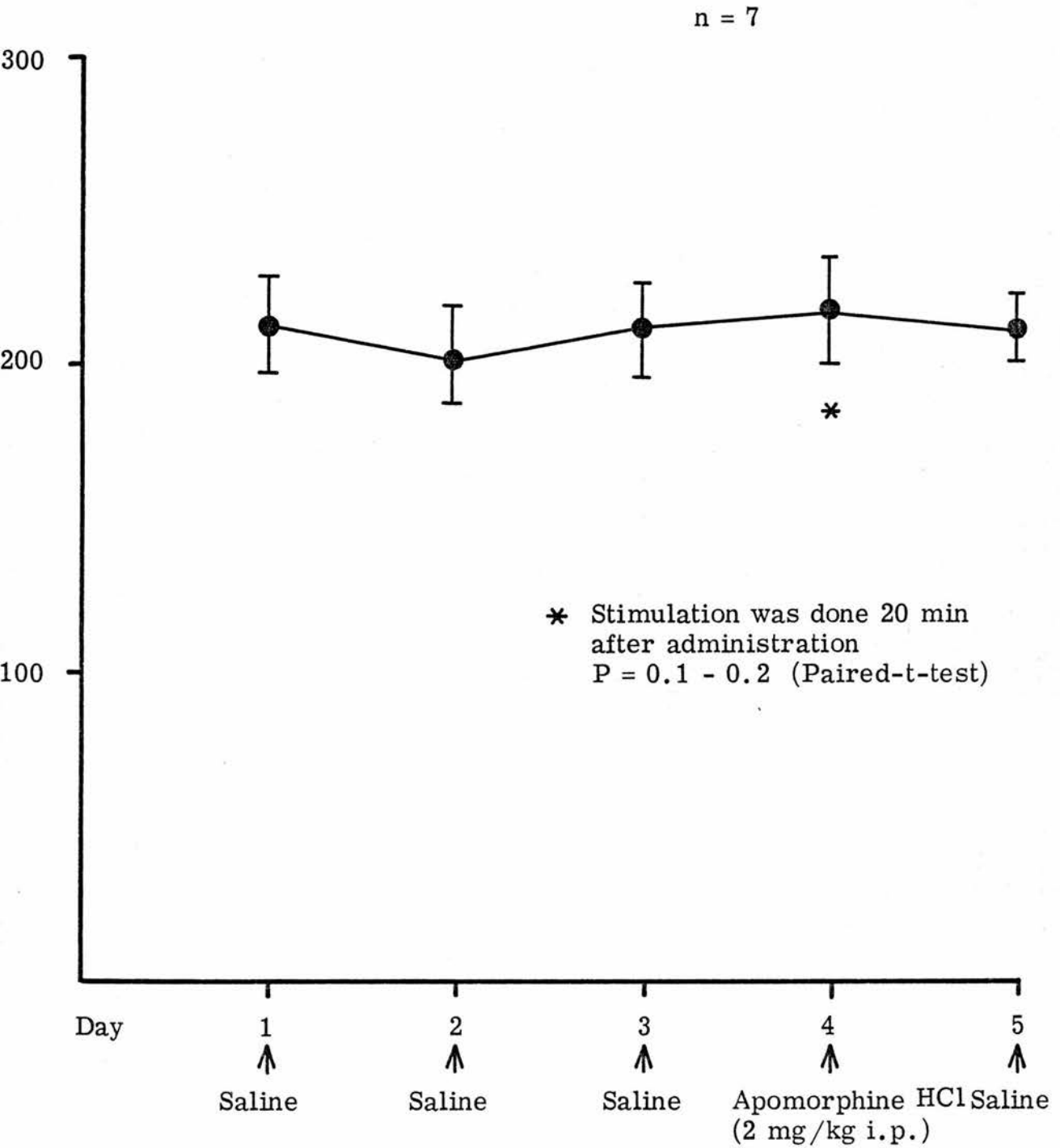
Fig 34A

Effect of bromocryptine (20 mg/kg i.p.) on the local after discharge threshold in rats kindled by amygdaloid stimulation.

n = 7



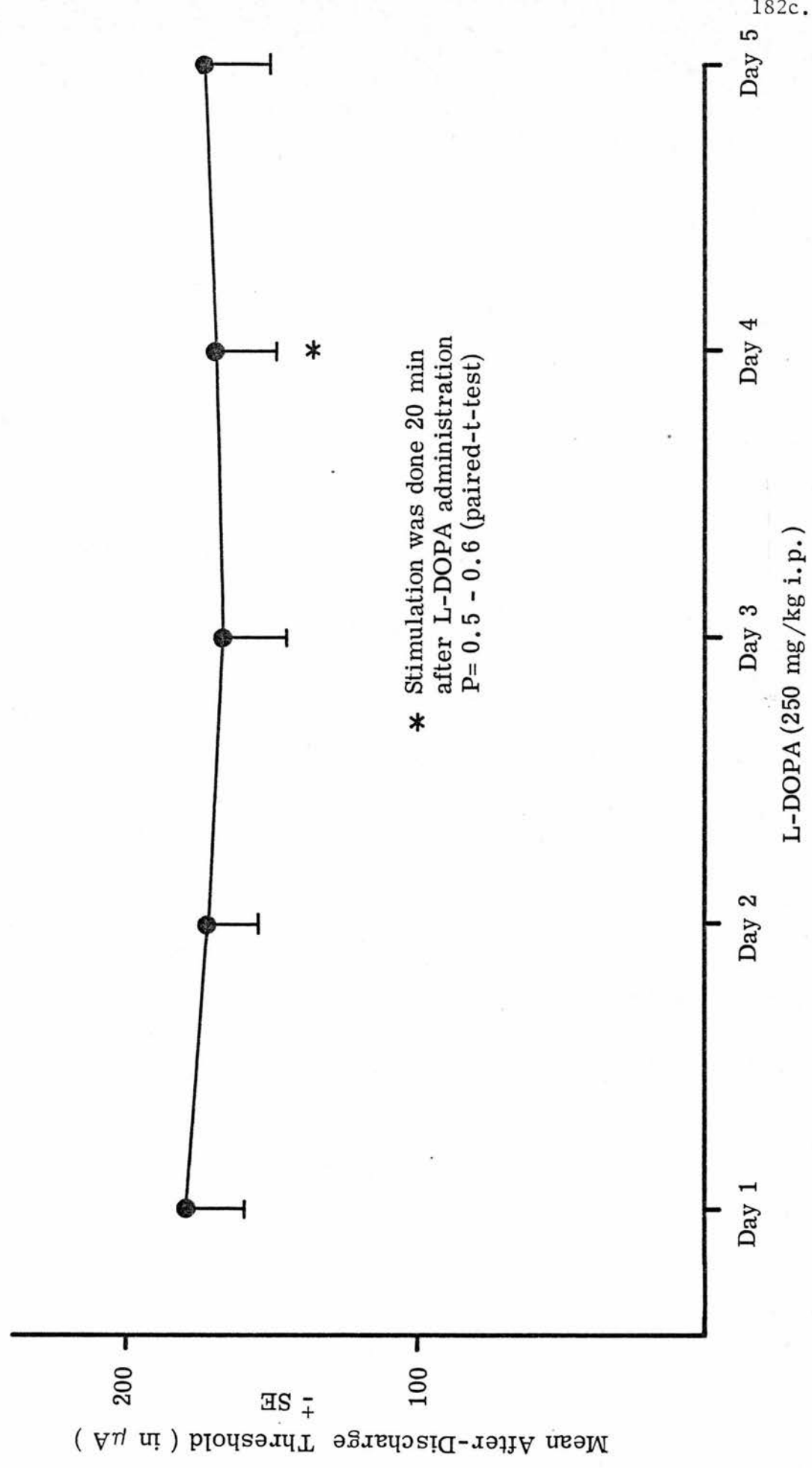
Effect of apomorphine (2 mg/kg i.p.) on the local after discharge threshold in amygdaloid kindled rats





Effect of L-DOPA (250 mg/kg i.p.) on the local after discharge threshold  
in rats kindled by stimulation of the amygdala

n = 6



the afterdischarge threshold.

On the fourth day, the threshold for afterdischarge was measured 4 hrs after bromocryptine, or 20 min after apomorphine or L-DOPA, and no significant change was found with either of these drugs (paired t-test).

With bromocryptine, 3 rats showed no change another 3 rats showed an increase of 20  $\mu$ A, while 1 rat showed a decrease of 20  $\mu$ A in the afterdischarge threshold (see Fig. 34A).

With apomorphine, 4 rats showed no change, 1 rat had a 20  $\mu$ A increase, while another 2 rats showed a 20  $\mu$ A decrease in the afterdischarge threshold (see Fig. 35A).

With L-DOPA 3 rats had no change, 2 had 20  $\mu$ A increase, while 1 rat had 20  $\mu$ A decline in afterdischarge threshold (see Fig. 36A).

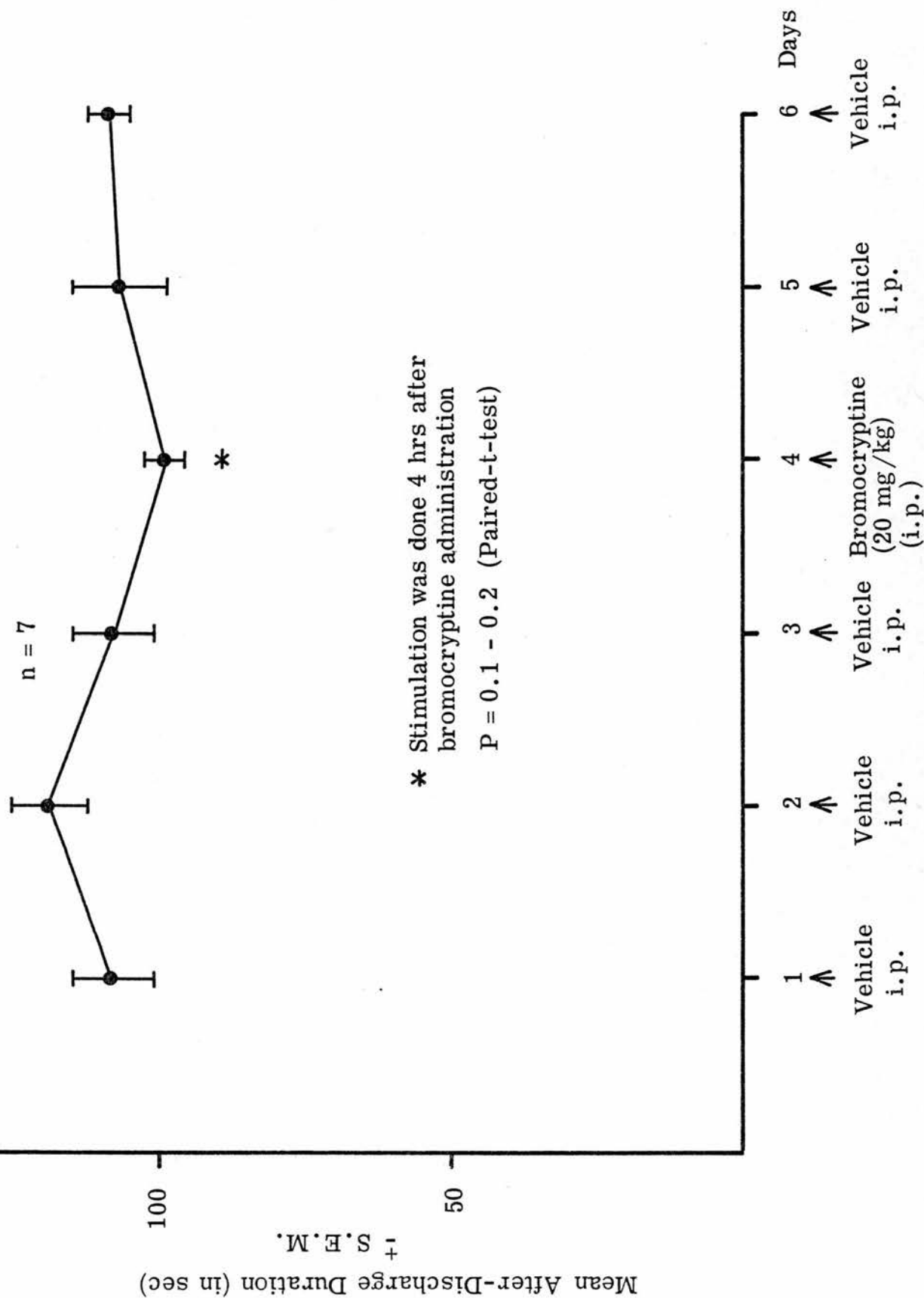
## 2. EFFECT ON AFTERDISCHARGE DURATION

The afterdischarge duration (in sec) in the above experiments was also measured. The afterdischarge duration during the first 3 days of stimulation was less stable than the afterdischarge threshold. In the 7 kindled rats in the bromocryptine experiment, the afterdischarge duration ranged between 80 sec and 156 sec, with a mean duration of 112 sec. In the 7 kindled rats of the apomorphine experiment, it ranged between 65 sec and 134 sec with a mean duration of 96 sec, while in the 6 kindled rats of the L-DOPA experiments, it ranged between 40 sec and 130 sec with a mean duration of 85 sec.

On the fourth day, the afterdischarge duration was also measured when the effect of dopamine agonists on behaviour (i.e. increased

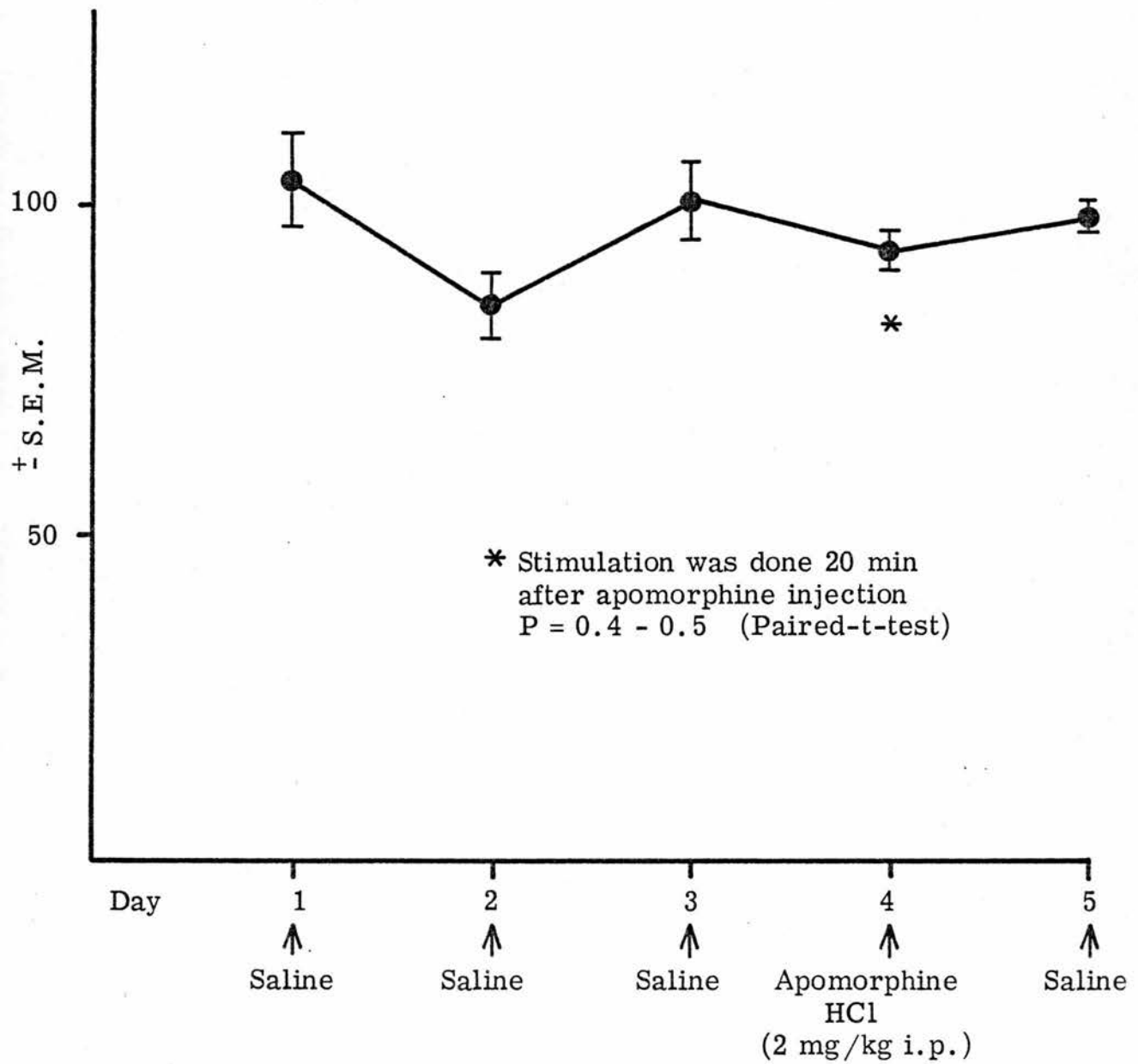
Fig 34B

Effect of bromocryptine (20 mg/kg i.p.) on the duration of after discharge in amygdaloid kindled rats.



Effect of apomorphine (2 mg/kg i.p.) on the after discharge duration  
in amygdaloid kindled rats

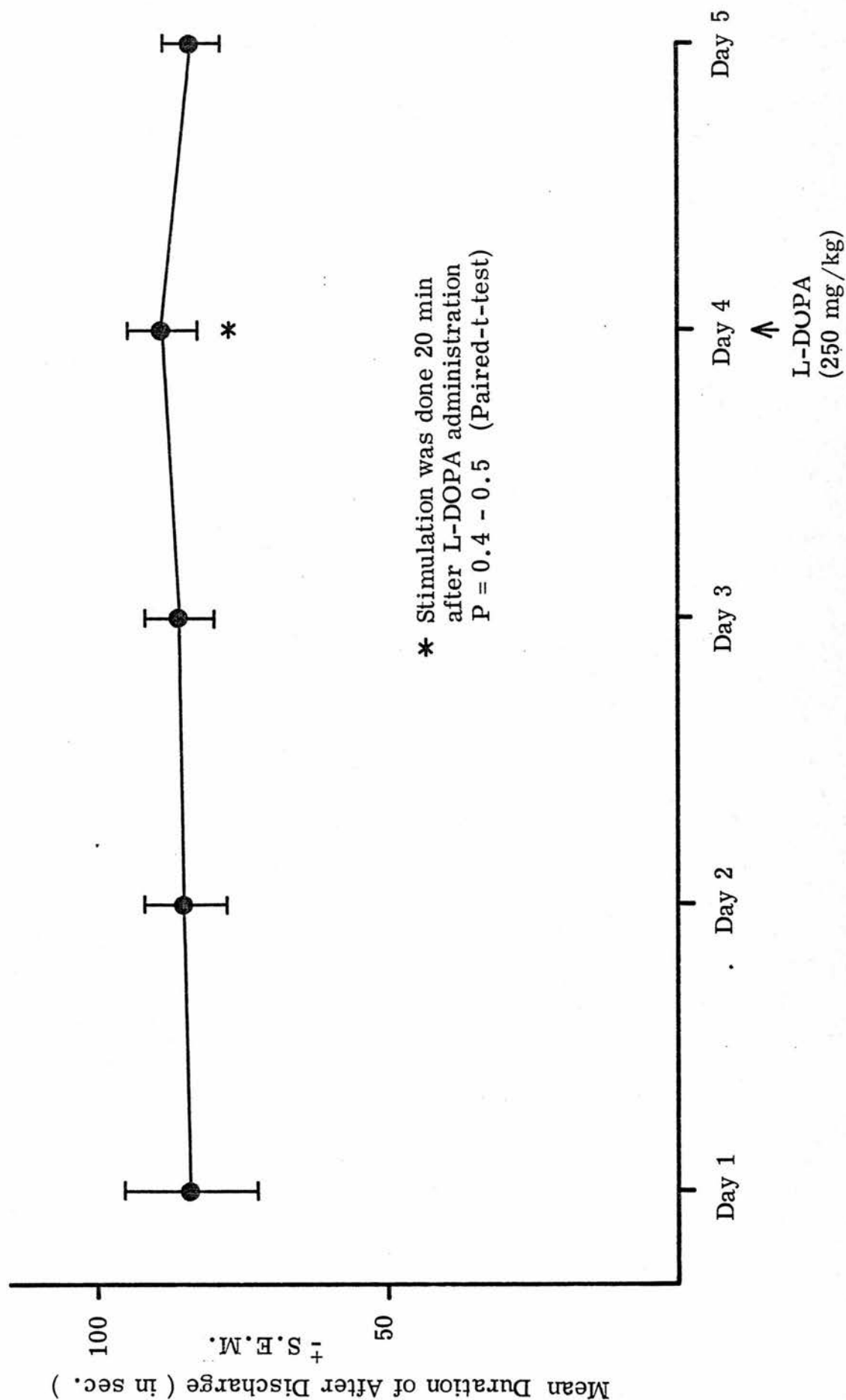
n = 7



Effect of L-DOPA (250 mg/kg i.p.) on the duration of after discharge  
in rats kindled by amygdaloid stimulations.

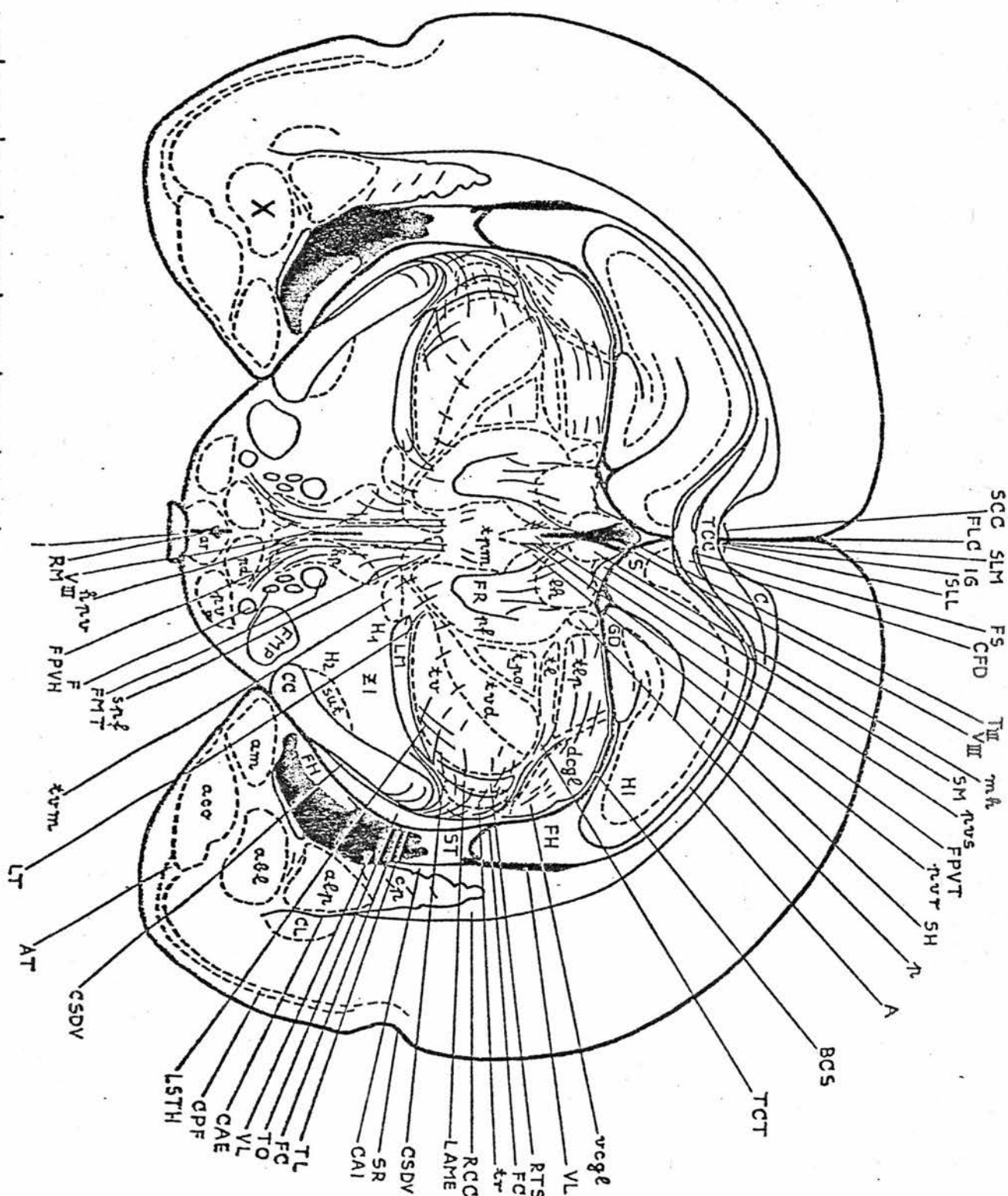
Fig 36B

n = 6



arousal, stereotypy and pileoerection) were marked. Similarly, these dopamine agonists produced no significant change in the duration of afterdischarge (paired t-test) (see Figs. 34B, 35B, and 36B).

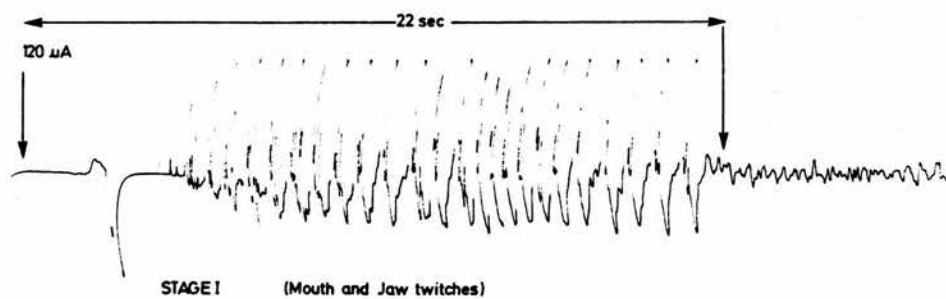
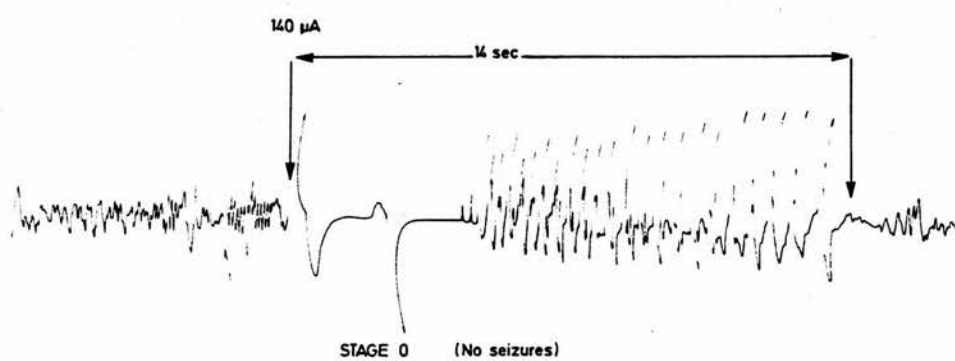
Fig. 37 X Site of electrode implantation into the basolateral region of the left amygdala (A 4.5 mm, L 4.4mm, V 7.9 mm), (Konig & Klippel, 1963).

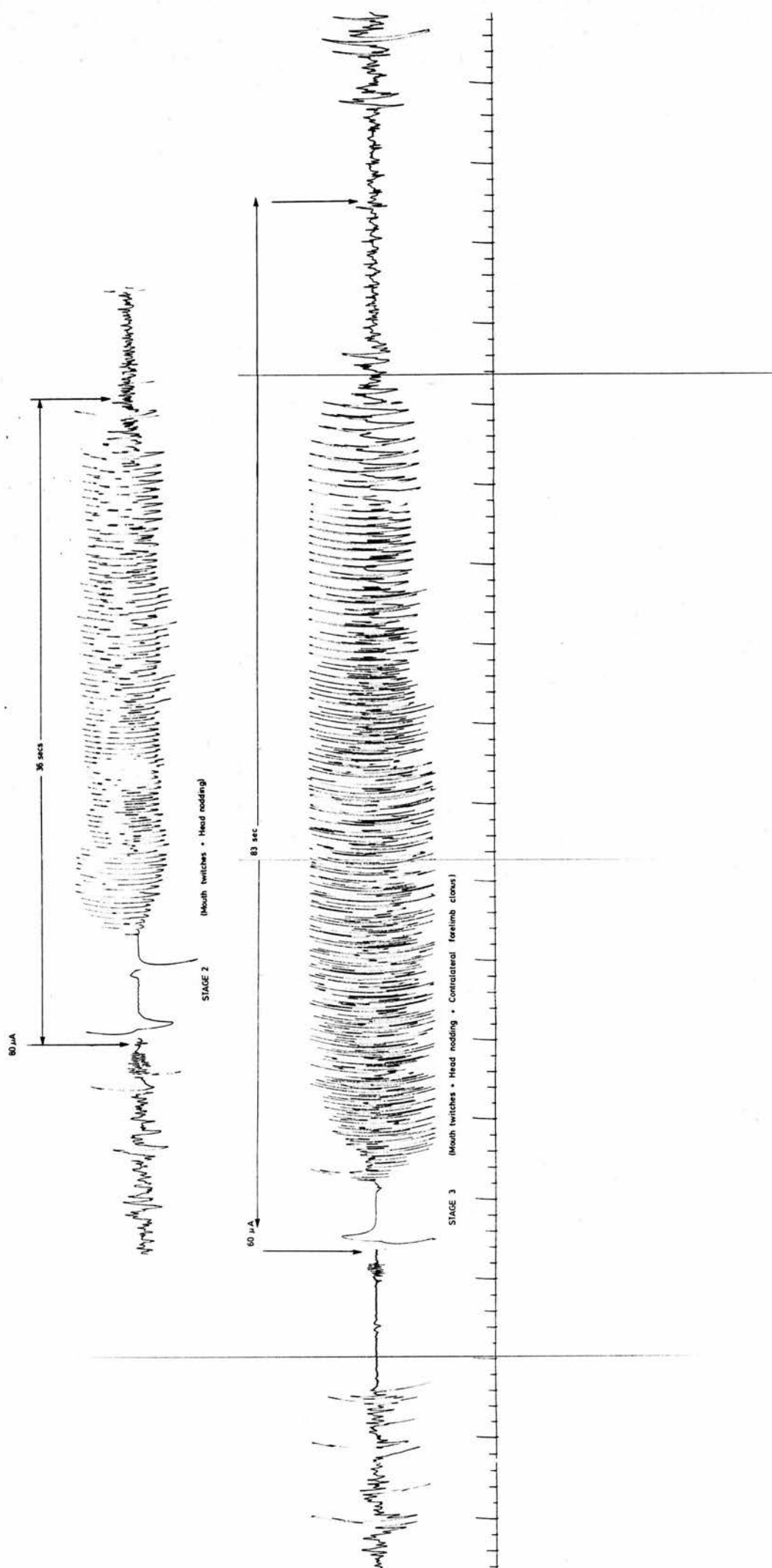


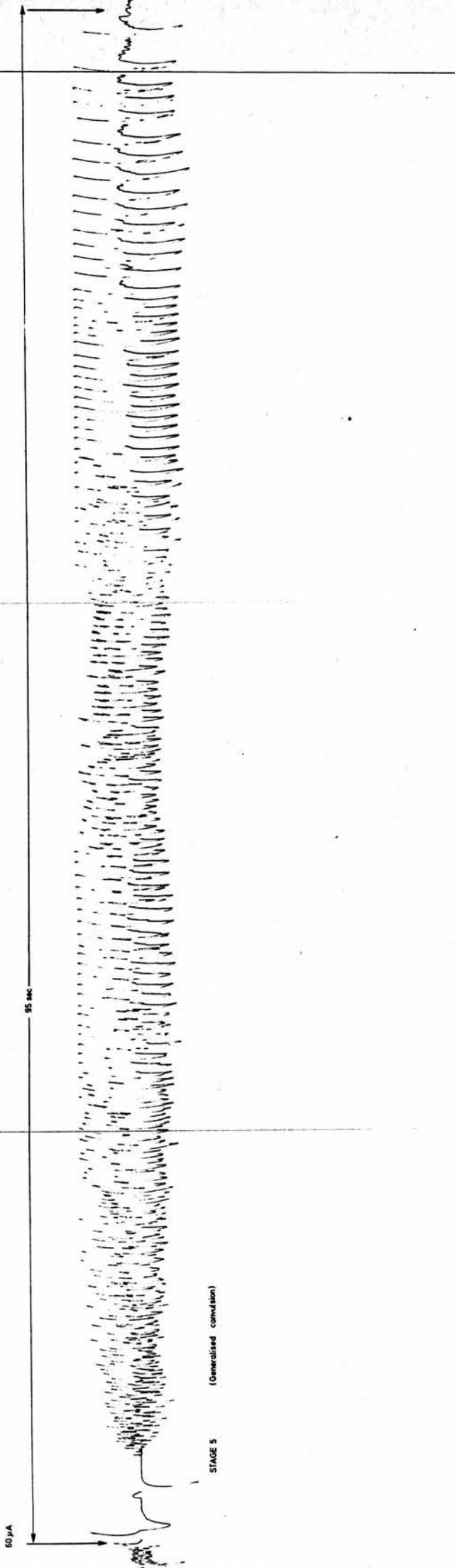
Legend to recorded afterdischarges from the amygdala

Samples of recordings from the stimulated amygdala of control rats showing the afterdischarge threshold (in  $\mu\text{A}$ ) and duration (in sec) during the different stages of development of amygdaloid kindling.









STAGE 5  
(Generalised convulsion)

## DISCUSSION

Electrographic seizure responses and behavioural convulsions could be induced by a number of experimental treatments (Purpura et al, 1969). Some of these treatments would result in permanent alteration in neural function, and these had been proposed as models for investigation into the mechanism of neural plasticity (Goddard et al, 1969, Morrel, 1969, Racine, 1972). One such treatment was kindling. At least two independent neural changes occurred in kindling. First, repeated electrical stimulation progressively lowered the threshold for epileptiform afterdischarge at the tip of the electrode, and secondly, once an afterdischarge was generated, it was propagated with increasing strength to other limbic and motor sites in the brain (Racine et al, 1972a).

### Afterdischarge threshold

In the present work, during the first few stimulations, the afterdischarge threshold in the stimulated amygdala was variable between different rats at one stimulation session, and in the same rat at different stimulations. However, later on in the course of kindling development, there was a progressive reduction in the threshold convulsive afterdischarge, and this was in agreement with the previous work (Racine, 1972a). This supported the assumption that one of the hallmarks of kindling was progressive reduction in the convulsive afterdischarge threshold.

### The spread of afterdischarge and the motor seizure

The sequence of motor changes induced by amygdaloid kindling included jaw movements, head nodding, forelimb twitches, rearing, and rearing with loss of postural control. This sequence was similar to that reported by

other workers (Racine, 1972b). In the present experiments, the duration of afterdischarge showed a progressive increase with the sequence of development of motor seizure. This reflected the increased intensity of spread of the afterdischarge from the site of stimulation to other motor sites in the brain; hence, the development of different stages of the motor seizure.

It had been shown that activation of the reticular formation occurred just before the appearance of generalized seizure in kindling (Wada & Osawa, 1975). Furthermore, electrical changes were detected in the motor cortex during the early stages of kindling. Similar generalized convulsions to those triggered by daily amygdaloid stimulation were elicited following stimulation of the prefrontal cortex in rats (Wada et al, 1974) suggesting that the cortex participated in kindling convulsions. However, experiments using aspiration lesions of the prefrontal and orbital cortex showed retardation of kindling, but did not prevent it, suggesting that these cortical areas were not essential for the development of generalized convulsions (McCaughran et al, 1975).

Previous studies showed that the forebrain commissures (corpus callosum, anterior commissure and hippocampal commissure) are involved in the propagation of epileptiform activity between various areas of the cerebral cortices (Erickson, 1940, Brodal, 1948, McCulloch & Garrol, 1941, Fox & Schmidt, 1943, Brodal, 1948, McCulloch & Garrol, 1941, Frost et al, 1958, Fox et al, 1948, Simpson, 1952). However, these pathways were found not critical for amygdaloid kindling in the rat, but they participated in determining the pattern of seizure development and propagation of afterdischarge to contralateral structures. Sectioning of the corpus callosum

and hippocampal commissure were reported to produce disruption of the development of bisymmetrical stage 5 seizure in kindling, and also caused a severe disruption in the propagation of afterdischarge to the contralateral frontal and motor cortices. However, the propagation of afterdischarge to the contralateral amygdala was attenuated only by bisection of all three commissures. Moreover, regardless of the extent of forebrain bisection, bilateral discharges were reported in the mesencephalic reticular formation (McCaughran et al, 1978a). Therefore, it was possible that limbic-brain stem connections might be more functionally involved in amygdaloid kindling than forebrain commissural pathways. Furthermore, lesion experiments showed that the non-specific thalamic regions did not participate critically in amygdaloid kindling (McCaughran et al, 1978b). Since previous studies established a major role for the non-specific thalamus in partial epilepsy of neocortical origin, then it could be suggested that the mechanism that underly seizures of neocortical origin may differ from those of limbic origin.

#### Catecholamines and acetylcholine in kindling

Several reports suggested cholinergic circuits were involved in seizure propagation (Jasper, 1969). Some workers have shown that the daily injection of subconvulsive doses of carbachol into the amygdala would eventually elicit motor seizures. Some workers showed that atropine retarded kindling in rats (Arnold et al, 1973), but this was not confirmed by others (Wada, 1975 - unpublished data). Depletion of central catecholamine neuro-transmitters by reserpine or 6-OHDA facilitated the electrographic and motor seizure responses evoked by repeated amygdaloid stimulation (Arnold et al, 1973, Corcoran et al, 1974). Moreover, intensification and prolongation of the local amygdaloid afterdischarge was reported

following reserpine administration (Wilkinson & Halpern, 1977). This evidence indicated that catecholamines are inhibitory in some parts of the central nervous system and may be inhibitory over some cholinergic circuits (Bloom et al, 1965, Jasper, 1969, Stein, 1968).

Tyrosine hydroxylase activity as index of catecholamine production in kindling.

It seems likely that catecholamine depletion facilitated the development of amygdaloid seizures by reducing the tonic inhibition within the limbic system. It was reported that the enduring focal sensitisation in kindling was attributed in part to a persistent reduction of post-synaptic inhibitory influences at the site of stimulation (Burnham & Gardner-Medwin, 1974). The reduced tyrosine hydroxylase activity, together with the reported lowered dopamine, but not noradrenaline content, in the stimulated amygdala suggested that a reduction of dopaminergic neurotransmission was involved during the initiation of focal postsynaptic hyperexcitability changes in amygdaloid kindling. However, this focal reduction of post-synaptic inhibition might involve other neurotransmitter systems in kindling initiated from other sites, such as the hippocampus which virtually lack dopaminergic innervation. Moreover, the presence of tissue necrosis at the electrode tip would have inhibited amygdaloid kindling (Goddard et al, 1969).

Furthermore, no change in tyrosine hydroxylase activity was found in the contralateral amygdala, hypothalamus as well as the frontal cortex and thalamus on both sides, which are involved in the spread of amygdaloid afterdischarge in kindled rats. Similarly, no significant alterations in enzyme activity was found in the hippocampus which is closely involved

in amygdaloid kindled afterdischarge (Wada & Sato, 1974), but incapable of producing generalized seizure when stimulated in kindled animals (Burnham, 1975). The absence of any significant change in catecholamine production in all brain regions studied, apart from the stimulated amygdala, does suggest that the mechanisms of spread of afterdischarge are different from those initiating the focal hyperexcitability in kindling convulsions. It could consequently be concluded at this stage that kindling induced a definite, long-lasting reduction in tyrosine hydroxylase activity at the site of stimulated amygdala.

It might be possible that reduction in tyrosine hydroxylase activity might reflect damage at the site of the implanted electrode. The lack of alteration in enzyme activity at a similar site in the sham-operated control rats would render this explanation unlikely. It is also unlikely that the low intensity current, used in the stimulation procedure, to have caused tissue damage. Tissue damage might reduce the local activity of other transmitter synthesizing enzymes besides that of tyrosine hydroxylase. However, no change in choline acetylase or glutamic acid decarboxylase were observed at the site of the stimulated amygdala in kindled rats in comparison to controls (Blackwood, 1976 - unpublished results). Moreover, no histological evidence of degeneration was reported in the stimulated amygdala in kindled rats (Brotchie et al, 1978) and no local evidence of tissue degeneration was seen at the site of stimulation in the present work with drug experiments.

#### Some dopamine agonists and kindling

To follow further on the dopamine system in kindling, the effects of dopamine agonists were assessed. Kindling had been related to systems



such as the amygdala, hippocampus, cingulate and frontal cortex, caudate-putamen, nucleus accumbens, olfactory bulb and the septum. All these areas were post-synaptic to catecholaminergic innervation. Therefore, it was possible that kindling seizures result from activation of systems that were normally inhibited by ascending catecholamine projections. This was in contrast to the self-stimulation procedure sites which contained either the cell bodies or axonal projection of the catecholamine system, and in which self-stimulation reward was not compounded with seizure development.

The dopamine agonists bromocryptine and apomorphine produced no significant effect on the rate of development of amygdaloid kindling. Furthermore, dopamine agonists had no effect on the threshold as well as the spread of afterdischarge. The lack of effect of dopamine agonists in kindling seemed paradoxical to the expected inhibitory role of dopamine in the amygdala (Ben-Ari & Kelly, 1976), and the reduced tyrosine hydroxylase activity seen in the present work. This was also in contrast to the anti-epileptic effect of dopamine agonists on cortical foci, reported in Section II of this thesis.

Activation of striatal neurons by dopamine agonists mediated at least in part the inhibitory effect of dopamine agonists on cortical epileptic foci induced by cobalt application (Section II). It had been shown that the caudate nucleus inhibited bioelectrical activity in several brain regions including the cerebral cortex (Amato et al, 1964) and amygdaloid nucleus complex (La Grutta et al, 1971), both of which possessed a low threshold for epileptic phenomena. The striatum gained access to the limbic system via the lateral habenular nucleus with which it was

connected by pallido-habenular fibers. Moreover, the hippocampus, amygdala and pyriform cortex project to islands of cells which were located anterior to the head of the caudate nucleus in the nucleus accumbens, and the nucleus stria terminalis. However, the ineffectiveness of dopamine agonists, as well as the unaltered tyrosine hydroxylase activity in the striatum suggested that the striatum neither inhibited nor did it participate in reducing the focal inhibition at the site of stimulation in the amygdala. It seemed that sensitization by low intensity stimulation of the amygdala caused alterations of the postsynaptic receptor population in the stimulated amygdala which rendered then unresponsive to striatal stimulation as induced by dopamine agonists.

#### Dopamine receptors and kindling

Dopamine receptors had been involved in the regulation of tyrosine hydroxylase activity and consequently dopamine synthesis and turnover by the dopaminergic neurons. A post-synaptic location of these regulating dopamine receptors assumed that their blockade inhibited a feed back neuronal loop which when activated reduced the activity of dopaminergic neurons (Carlsson & Lindquist, 1963, Bunney et al, 1973a and b). An alternative hypothesis assumed that the dopamine receptors which regulated the activity of tyrosine hydroxylase were located presynaptically at dopaminergic terminals (autoreceptors). Without involvement of intermediate synaptic relay (Kehr et al, 1972) small doses of dopamine agonists bind to those autoreceptors in producing their inhibitory behavioural effects, as well as reducing the activity of tyrosine hydroxylase. Larger doses, however, bind to post-synaptic receptors in producing their enhanced behavioural effects as well as effecting a further reduction in tyrosine hydroxylase activity (Carlsson et al, 1976).

Some workers suggested that dopamine sensitive adenyl cyclase located in dopaminergic axonal terminals regulated the kinetic state of tyrosine hydroxylase in the striatum. This view was based on in vitro findings that cyclic adenosine monophosphate (c AMP) increased dopamine synthesis when added in high concentrations to striatal slices or synapotosomes, probably by increasing the affinity of tyrosine hydroxylase to its pteridine co-factor (Harris et al, 1974). However, in vivo experiments failed to support this theory (Zivkovic et al, 1975). It is possible that the state of membrane polarization or the frequency of membrane polarity changes might influence the kinetic state of tyrosine hydroxylase through a change in the cyclic AMP content in the nerve terminal cytosol.

The reduction of dopamine content in the stimulated amygdala in kindled rats reported by Engel & Sharpless (1977) could be due to increased dopamine release and inactivation or to reduced catecholamine production. The finding of reduced tyrosine hydroxylase activity in this region supported the latter possibility. Furthermore, since the noradrenaline content was reported to be normal in the stimulated amygdala, it seemed that this reduction affected the enzyme activity in dopaminergic neurons. This manifested itself as about 30% reduction in total enzyme activity in catecholaminergic terminals in the stimulated amygdala as measured by the present method of assay, in comparison to the contralateral side and to controls.

It could be possible that repeated stimulation by low intensity current affected reduction in tyrosine hydroxylase activity and dopamine production by stimulating the pre-synaptic dopamine autoreceptors and/or affecting the polarization of axonal membrane. Activation by the

stimulating current of post-synaptic and/or internuncial neurons involved in the post-synaptic feedback neuronal control of dopamine synthesis might also be a contributory factor. It is well known that neuronal activity at any one time depends on the net balance exerted upon it by inhibitory and excitatory influences (Goddard et al, 1969). Since dopamine is an inhibitory transmitter in the amygdala (Ben-Ari & Kelly, 1976), then a reduction in dopaminergic neuro-transmission post-synaptically would contribute, at least in part, to the persistent hyperexcitability changes in post-synaptic neurons at the site of stimulation. These changes would be manifested by lowering of the afterdischarge threshold between successive stimulation, as well as the development of interictal discharges between stimulations. Thus, the reduction of tyrosine hydroxylase activity could be secondary to alteration in pre-synaptic events with more contribution from post-synaptic feedback mechanisms as the post-synaptic neurons become progressively hyperexcitable with possible alteration in receptor mechanisms resulting from repeated stimulations.

However, if reduced dopamine release and transmission was the only factor, then it would be expected for dopamine agonists to exert some inhibitory effect at the post-synaptic receptors, which is not the case. It might be worth, at this stage, to describe the dopamine receptor binding in the amygdala.

Using [ $^3\text{H}$ ] dopamine and [ $^3\text{H}$ ] haloperidol it was shown that [ $^3\text{H}$ ] haloperidol binding in the amygdala was about 30% of that of the striatum and 50% of that of the olfactory tubercle, while [ $^3\text{H}$ ] dopamine binding sites were much less abundant (Creese et al, 1975). This suggested that dopamine receptor binding in the amygdala existed mainly in the antagonistic state. Thus, it might be possible that repeated sensitization with low intensity

current could have altered the structural configuration and/or reduced the agonist binding sites post-synaptically so as to interfere with their binding or response to endogenous or exogenous dopamine and dopamine agonists. This could also explain the reported lack of effect of amphetamine on amygdaloid afterdischarge in kindling (Kamei et al, 1977). It could also be possible that the antagonistic state of the dopamine receptors had been augmented by repeated stimulation. This assumption might be supported by the finding that the dopamine receptor blockers, chlorpromazine and haloperidol, increased the duration of afterdischarge and reduced its threshold in amygdaloid kindled rats (Kamei et al, 1977, Schmitt, 1967).

It is well known that if kindling stimulations were continued, spontaneous convulsions would be initiated (Goddard et al, 1969). This might be due to severe reduction in post-synaptic inhibition associated with marked hyperexcitability of post-synaptic membranes which might lower the threshold for afterdischarge to the extent that normal excitatory impulses arriving at the hyperexcitable synapses would initiate epileptic changes and thus motor seizure. These impulses could be mediated by cholinergic and possibly noradrenergic neurotransmission. Moreover, it might also be possible that changes of local post-synaptic receptor mechanisms might lead to the acquisition of self-stimulating properties by post-synaptic neurons. Some workers have already suggested that in any kindling process, an excitatory-inhibitory coupling, together with excitatory self-stimulation should be searched for (Morrel & Tsuru, 1976, Liebllich & Amari, 1978).

Another important feature is the facilitation of synaptic transmission

which accounted for the spread of afterdischarge in multiple neuronal circuits in kindling. This was referred to as enhanced electrical fluidity. At pathologically high levels of electrical fluidity, an electrical event occurring at any point in a neuronal matrix would be transmitted throughout that matrix, such that every cell would be stimulated by every other cell. It might also be that the learning process and memory consolidation resulted from a controlled increase of electrical fluidity in specific neuronal circuits, thus mimicking the kindling phenomenon to some extent.

It was reported that tricyclic anti-depressants inhibited the local afterdischarge in kindling. These drugs would inhibit the active re-uptake of the released catecholamines and 5-HT into the axonal terminals. It could be possible that their effect was mediated by 5-HT mechanisms, since the amygdala is also known to contain high concentrations of this neurotransmitter. However, there is no evidence yet of any specific alteration in 5-HT neurotransmission in amygdaloid kindling.

#### Functional linking hypothesis and kindling

Some workers described a first approximation model which accounts for the strongest phenomena defining kindling (Lieblich & Amari, 1978). This model was based on excitation-inhibition coupling of neural aggregates, to which a self-stimulation element of the excitatory aggregate was added. The functional linking hypothesis viewed the representation of kindling as a process of gradual transition through structural changes from a stable system to a system showing stability for small perturbations and oscillatory orbit for larger perturbations, and finally to a persistent purely oscillatory system. The anatomical linking hypothesis considered



the main neuronal systems involved in the kindling phenomenon to be located in the amygdaloid pyriform complex (where kindling is initiated), the hypothalamus, and the hippocampal-septal-preoptic system, respectively. It is well known that the amygdala is connected via the central amygdalofugal pathway and stria terminalis to the hypothalamus, the inhibitory aggregate representing the hippocampal-septal-preoptic system and the self-stimulating element of the excitatory aggregate as representing the amygdaloid pyriform complex.

Stimulation of the hypothalamic aggregate (or reducing its threshold) in this system retarded kindling from the amygdaloid aggregate, while reduction of its stimulation potentiated kindling. In addition, the model showed that kindling would not be possible from the hypothalamus even with drastic reduction of threshold. Moreover, Callaghan & Schwark (1975) demonstrated a reduction in noradrenaline content in the hypothalamus of kindled rats. However, tyrosine hydroxylase activity in the region showed some reduction in the present work, but this was not significant. Moreover, it is known that stimulation of the amygdaloid complex produces afterdischarge in the hypothalamus. The model also showed that stimulation of the inhibitory hippocampal-septal-preoptic system inhibited and stabilised kindling, while moderate elevation of its threshold reduced this inhibition and facilitated kindling. Thus, it is possible for such reduction in inhibition by this system to occur with repeated amygdaloid stimulation, and in effect, this would enhance synaptic transmission within and outside the stimulated area. This factor, together with the development of hyperexcitability changes and possibly the self-stimulation properties in the stimulated amygdala, might explain the enhanced spread of afterdischarge following repetitive stimulation as well as the development of spontaneous

convulsions on continuing stimulation.

#### Dopamine agonists and cerebral blood flow

Dopamine and dopamine agonists, like apomorphine, are known to increase the cerebral blood flow, oxygen uptake and metabolic activity of cortical neurons, which effects were blocked by pre-treatment with pimozide. These changes were also seen in areas that are lacking in dopamine receptors such as the cerebellum (Teasdale & McCulloch, 1977, McCulloch & Harper, 1977). The absence of any significant effect of dopamine agonists in kindling, and their profound anti-epileptic effect on cortical foci in cobalt-implanted rats suggested that the mechanism of action of these drugs was due to dopamine receptor stimulation without a definite contribution from increased regional cerebral circulation which they could induce. Furthermore, a reduction in cerebral blood flow was detected in some atrophic cortical epileptic foci in man (Lavey, Melamed, Portnoy & Carman, 1977), while increased vascularity was seen in others.

#### Discharge and spread - possible mechanisms

It seemed from the present and previous work that the small amount of dopamine in the amygdala was important in the control of neuronal excitability of this region. It was possible that a similar mechanism would apply to other sites that were susceptible to the development of kindling convulsion. Disturbances leading to a reduction of its inhibitory influence at the post-synaptic receptors would facilitate the initiation or development of hyperexcitability changes and afterdischarge response to minimal stimuli. These stimuli repeated at optimal intervals might culminate in the development of generalized convulsions resulting from the spread of afterdischarge to secondary sites.



The lack of changes in catecholamine production at sites other than the stimulated amygdala would suggest that non-catecholaminergic mechanisms were involved in the spread of afterdischarge from the site of stimulation. Cholinergic transmission might be a candidate for such a mechanism of spread. However, the results of inhibiting cholinergic transmission with atropine were diverse (Arnold et al, 1973, Wada, 1975) in postulating that it would delay the propagation of kindling afterdischarge, but activation of the cholinergic reticular formation is known to occur prior to the development of generalized seizures in kindling,

Inhibition of multi-synaptic transmission in this reticular system might well explain the prophylactic value of diazepam in this model.

Moreover, there is evidence that gabbaminergic transmission might be involved in the mechanism of seizure spread. Valproic acid is known to increase the concentration of brain gamma aminobutyric acid (GABA) perhaps by inhibiting the enzyme GABA-transaminase (Godin et al, 1969, Smiler et al, 1973). Valproic acid was found to retard the development of kindling. However, it did not prevent the propagation of afterdischarge element to the frontal cortex. Moreover, valproic acid was found to prevent the generalization of seizure, and in large doses to inhibit seizures, altogether in kindled rats (Leviel & Naquet, 1972). Some workers suggested that GABA counteracted the reduction in the afterdischarge threshold induced by kindling stimulation (Tanaka & Lange, 1975). Moreover, since the increase in GABA levels did not prevent focal seizures, and it allowed the electrographic element of afterdischarge to reach the cortex, it seemed that GABA acted by preventing the transformation of simple focal discharge into generalized motor seizure. This, again, shows that the mechanism underlying the focal afterdischarge and the secondary generalized seizure are essentially different. The finding of reduced tyrosine hydroxylase

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activity in the stimulated amygdala and absence of significant alteration in catecholamine production in other brain regions studied support this assumption further.

### Kindling and learning

The fact that kindled behaviour alterations were persistent and might be permanent (Wada et al, 1974) led to the speculation that kindling and learning had certain basic mechanisms in common (Gaito, 1974, Goddard & Douglas, 1975, Leech & McIntyre, 1976, Zaide, 1974). The present finding of tyrosine hydroxylase activity as well as the previous findings of local dopamine changes might reveal a plastic property of the catecholamine projections that could also be invoked to explain aspects of certain acquired alterations in behaviour. It is well known that memory and learning involved inhibitory mechanisms (Eccles, 1973), which could take the form of selectively decreased inhibitory synaptic activity. The catecholamine system was probably involved in such function due to its diffuse projections (Fuxe 1965, Jacobowitz & Palkovits, 1974, Lindval & Bjorklund, 1974, Sato & Nakashima, 1975, Ungerstedt, 1971), and their plastic properties (Katzman et al, 1971, Moore et al, 1972, Wada & Sato, 1974, Wada et al, 1974). The involvement of catecholamine systems in some learning experiments has already been put forward (Nikiforov & Knyazeu, 1975).

## CONCLUSIONS

HMPG and HVA levels, reflecting noradrenaline and dopamine turnover respectively, were estimated in the frontal cortex and striatum of cobalt-implanted epileptic rats. A micromethod was used for this purpose. This method was precise, specific, and sensitive for the simultaneous measurement of both metabolites in the small amount of tissue of rat brain. Previous reports showed dramatic reduction of tyrosine hydroxylase activity and compensatory reduction in activity of catecholamine catabolising enzymes in cortical foci and striatum in this model.

No significant change in the concentration of either metabolite was found in the brain regions studied. It was suggested that impairment of the saturable transport mechanism and possibly of the enzymatic conjugation of these metabolites due to cellular degeneration and/or cobalt ion enzyme toxicity prevented the expected reduction in their concentrations in those brain regions. It was then concluded that changes in the concentration of these major catecholamine metabolites did not show and even could reflect opposite changes of their respective amine turnover in the presence of tissue degeneration.

Post-mortem studies on brain tissue showed that HVA is much more stable than HMPG under different conditions after death. This was possibly due to the greater affinity of the catabolising enzyme for intermediate catabolites of noradrenaline than those of dopamine, hence contributing to higher HMPG levels under some of these conditions. These factors might have contributed to the high HMPG levels previously reported following cobalt application.

Recent reports in this model in immuno-suppressed animals showed that cobalt ion is capable of producing

epileptic changes before the development of substantial cortical or sub-cortical necrosis or biochemical changes; thus, it seemed that the biochemical findings in the present and previous works were more due to the tissue degeneration than to the toxic effect of cobalt on enzyme systems associated with catecholaminergic neurotransmission.

It also seemed that the earlier onset of cellular degeneration in cortical foci with possible local denervation supersensitivity facilitated the development of epileptic changes. The normal concentration of noradrenaline in epileptic foci, and paralleled by similar findings in the striatum, could have contributed to the neuronal hyperexcitability in this model.

To elaborate more on the role of the dopamine system in the cobalt model of epilepsy, the initial work of Ashcroft et al (1974) was extended employing different agonists and antagonists. It was found that apomorphine, bromocryptine and lisuride all suppressed epileptic activity in a dose-dependent manner. Lisuride was most potent, followed by apomorphine, bromocryptine and the least potent was CF 25-397. The chronic administration of bromocryptine attenuated the development of epileptic changes, particularly in the secondary foci. Pimozide, the dopamine receptor antagonist, potentiated the epileptic activity in cobalt rats, and also prevented the anti-epileptic effect of dopamine agonists. This is in contrast to the lack of effect of the anti-cholinergic drug, hyoscine, previously observed.

Similar anti-epileptic effect for dopamine agonists was also seen in other forms of sensory epilepsy including the reflex epilepsies and the later stages of electroshock seizures.

The intrastriatal administration of dopamine or apomorphine desynchronized the epileptic cortex while destruction of catecholamine terminals in striatum potentiated the epileptic changes.

There is evidence that proprioceptive impulses arising from motor activity play an important role in the evolution of seizure response in sensory models of epilepsy which include, in addition to the reflex epilepsies, the focal motor epilepsy such as that induced by cobalt application to frontal cortex. Thus, it is possible that dopamine agonists might act directly or indirectly on this recurrent system.

Moreover, since dopamine receptors are not found in the sites of the primary and secondary foci in frontal cortex, it was suggested that dopamine agonists induced their anti-epileptic effects, at least in part, by stimulation of striatal dopamine receptors, in the following ways:

- a. increasing the level of arousal by stimulation of ARAS, and
- b. modulating the activity of the motor cortex.

These effects were probably mediated by fiber connections of the striatum with the reticular formation and motor cortex from which the pyramidal tract originates, as well as through descending extrapyramidal pathways such as the reticulospinal and tectospinal tracts.

The inhibition of epileptic activity in both cortical epileptic foci following unilateral intrastriatal injection of dopamine was possibly mediated by fiber connections between the striatum on both sides via the thalamus, and then with the motor cortex. This also showed the importance of the thalamus as a relay station in desynchronizing cortical epileptic



activity, in addition to its reported participation with the corpus callosum in the propagation of epileptic discharge from the primary focus as well as its synchronizing effect on cortical epileptic activity.

The role of the dopamine system in subcortical epileptic foci induced by kindling stimulation was also studied. Previous reports showed the inhibitory role of catecholamines in amygdaloid kindling.

In the present work, tyrosine hydroxylase activity was found to be reduced in the stimulated, but not the contralateral, amygdala or in other brain regions involved in the spread of afterdischarge in amygdaloid kindled rats. The reduction of tyrosine hydroxylase activity and dopamine production could be secondary to repeated alteration of presynaptic events by the stimulating current, with more contribution from post-synaptic neurons on becoming progressively hyperexcitable with repeated stimulations.

Since noradrenaline content was reported to be unchanged in the stimulated amygdala, it is possible that the reduction affected the enzyme activity associated with dopaminergic neurons. This was reflected as 30% reduction in the total enzyme activity controlling the biosynthesis of both catecholamines, as measured in the present method and compared to that of the contralateral amygdala and controls. It was reported that the focal sensitization in kindling was associated with the reduction of post-synaptic inhibitory influences at the site of stimulation. Dopamine was reported to be an inhibitory transmitter in the amygdala. The reduced tyrosine hydroxylase activity, together with the reported lowered dopamine, but not noradrenaline, content in the stimulated amygdala suggested that a reduction of dopaminergic neurotransmission was involved during the development of focal hyperexcitability changes in amygdaloid kindling. However, this focal inhibition might involve other neurotransmitter systems in the

kindling initiated from other sites such as the hippocampus which virtually lack dopaminergic innervation.

Dopamine agonists produced no significant effect either on the rate of kindling development or on the afterdischarge threshold and duration in kindled rats. This finding together with the unaltered tyrosine hydroxylase activity in the striatum suggested that the striatum neither inhibited nor did it participate in reducing the focal inhibition at the site of stimulation in the amygdala. This occurred in spite of the presence of fiber connections allowing access of the striatum to the limbic system. Thus it seemed possible that sensitization by low intensity brain stimulation caused alterations in the number or structural configuration of the local receptor population in the stimulated amygdala which rendered them unresponsive to striatal stimulation as induced by dopamine agonists.

The ineffectiveness of dopamine agonists might also be explained by the presence of dopamine receptor binding mainly in the antagonistic state. This state might have been perpetuated by the kindling sensitization procedure, possibly at the expense of agonists binding sites. The fact that haloperidol was reported to potentiate the spread of afterdischarge in amygdaloid kindled rats supports this assumption. It is also possible that sensitization with low intensity current altered the number or structure of agonist binding sites of the dopamine receptors which would render them unresponsive to exogenous or endogenous dopamine or dopamine agonists.

It is also suggested that the mechanisms involved in the initiation of afterdischarge (which was implicated with reduced dopaminergic transmission) are different from those involved in its spread to other brain regions, which could involve cholinergic and/or gabbaminergic neuro-



transmission in kindling. It is also possible that reduction in inhibition by the hippocampal-septal-preoptic system to have contributed at least in part to the persistence of hyperexcitable changes in the stimulated amygdala as well as enhancement of synaptic transmission and spread of afterdischarge in amygdaloid kindling.

It is also possible that repeated electrical stimulation might have led to the acquisition of self-stimulation properties by the hypersensitive post-synaptic neurons in the stimulated amygdala. This might explain the reported development of spontaneous convulsions with continuation of stimulation in kindled rats.

It is hoped that the above work provided some information on the role of the dopamine system in cortical as opposed to some subcortical foci of epilepsy.

It is speculated that some dopamine agonists like bromocryptine might be useful anti-convulsants in the future. It is also thought that dopamine receptor binding studies are indicated in kindling. Moreover, elucidation of the effectiveness of 5-HT transmission in inhibiting amygdaloid kindling might also be required.

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## Reduction in tyrosine hydroxylase activity in the rat amygdala induced by kindling stimulation

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Kindling, as described by Goddard et al.<sup>7</sup> involves electrical stimulation of certain brain structures, in particular the amygdala in the limbic system, until a previously ineffective stimulus comes to elicit a full motor seizure. Kindling has become an important experimental model for the study of epileptogenesis<sup>14,17</sup>. However, the mechanisms underlying kindling are still largely unknown. Some studies have suggested that catecholamine in the brain are inhibitory to kindling<sup>1</sup>, and to seizures in general<sup>13</sup>. It has been shown that cortical noradrenaline and dopamine levels are reduced in the brain of kindled cats<sup>15</sup>. Furthermore, it has been suggested that dopamine is an inhibitory transmitter in the amygdala<sup>2,12</sup>. More recently, it has been shown that unilateral lesion of the stria terminalis which contains the catecholamine-inhibitory afferents to the amygdala facilitates the early stages of development of kindling induced by ipsilateral amygdaloid stimulation in rats<sup>5</sup>. Moreover, the dopamine content was found to be locally reduced at the site of the stimulated amygdala in kindled rats<sup>6</sup>. However, other workers have reported a reduction in noradrenaline content in the hypothalamus but found no change in brain dopamine levels in kindled rats<sup>4</sup>.

In the present study, the activity of the enzyme tyrosine hydroxylase, the rate-limiting step of catecholamine biosynthesis<sup>11,16</sup>, was measured in the amygdala and other brain regions in rats kindled by amygdaloid stimulation.

Twenty-one male Wistar rats, 180–200 g in weight, were used for this study; 7 of these rats served as unoperated controls. Under halothane anaesthesia, the left basolateral amygdalae of 14 rats were stereotaxically implanted with bipolar stimulating electrodes of nichrome wire. The coordinates were: 4.5 mm anterior to the interaural line, 4.4 mm lateral to the midline, and 7.9 mm deep to the cortical surface<sup>10</sup>. Rats were allowed one week for recovery and daily stimulation was begun on the eighth day. The rats were divided into two groups. Seven were stimulated and 7 were non-stimulated sham controls. To the stimulated rats, a suprathreshold constant current, ranging between 100–300  $\mu$ A, and consisting of trains of 1 msec biphasic square wave pulses at a frequency of 60 Hz, was delivered for one second daily via the stimulating electrode

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using a constant current physiological stimulator (Farnell). Electrical activity was recorded from the amygdala on a Grass polygraph (model 7) before and after stimulus delivery and the duration of the ensuing afterdischarge was measured. Daily stimulation was continued until a generalised convulsion developed<sup>14</sup> on 3 consecutive days. The 7 experimental rats demonstrated afterdischarges on the first day of stimulation, and developed generalised convulsions between 9 and 13 days. The pattern of development of kindling did not differ from that reported by other workers<sup>7,14</sup>.

For weeks after the last stimulation, rats were killed by decapitation and their brains dissected out quickly on a cold stage at 4 °C. The following dissection was done with the aid of the dissecting microscope. The entire hippocampus was carefully dissected out on both sides of the brain. Two vertical cuts were then made from the inferior surface of the brain; the first at the level of the optic chiasma (A 5500  $\mu\text{m}$ )<sup>10</sup> and the second at the level of junction of the cerebral peduncles with the base of the cerebral hemispheres (A 3000  $\mu\text{m}$ )<sup>10</sup>, thus dividing the brain into 3 parts. From the anterior part, tissue portions were taken from the frontal cortex and striatum on both

TABLE 1

*Tyrosine hydroxylase activity nmole [<sup>3</sup>H]L-DOPA formed/h/g wet wt.) in different brain regions in amygdaloid kindled rats*

Each value represents the mean activity  $\pm$  S.E. (n).

	Right	Left
Amygdala		
Kindled (7)	1.96 $\pm$ 0.13	1.31 $\pm$ 0.09*,**
Sham-operated (7)	2.11 $\pm$ 0.17	2.10 $\pm$ 0.15*
Unoperated (7)	1.95 $\pm$ 0.10	1.98 $\pm$ 0.07
Hippocampus		
Kindled (6)	0.36 $\pm$ 0.02	0.34 $\pm$ 0.04
Sham-operated (6)	0.38 $\pm$ 0.03	0.42 $\pm$ 0.02
Unoperated (6)	0.38 $\pm$ 0.02	0.40 $\pm$ 0.03
Hypothalamus		
Kindled (6)	4.17 $\pm$ 0.28	3.58 $\pm$ 0.28
Sham-operated (6)	4.31 $\pm$ 0.19	4.45 $\pm$ 0.25
Unoperated (6)	5.01 $\pm$ 0.18	4.89 $\pm$ 0.27
Thalamus		
Kindled (6)	1.13 $\pm$ 0.11	0.82 $\pm$ 0.16
Sham-operated (6)	0.89 $\pm$ 0.10	0.88 $\pm$ 0.10
Unoperated (6)	1.10 $\pm$ 0.18	0.92 $\pm$ 0.16
Striatum		
Kindled (6)	23.99 $\pm$ 1.05	21.79 $\pm$ 0.68
Sham-operated (6)	24.02 $\pm$ 1.63	24.62 $\pm$ 1.24
Unoperated (6)	23.99 $\pm$ 1.66	25.14 $\pm$ 1.85
Cortex		
Kindled (7)	0.28 $\pm$ 0.01	0.26 $\pm$ 0.02
Sham-operated (7)	0.27 $\pm$ 0.02	0.27 $\pm$ 0.02
Unoperated (7)	0.27 $\pm$ 0.02	0.28 $\pm$ 0.02

\* Site of electrode implantation into the left amygdala.

\*\*  $P < 0.002$  for comparison of right and left sides (Mann-Whitney U-test)

sides. From the middle part, both amygdalae were dissected out under the dissecting microscope. Tissue portions from the hypothalamus and thalamus were then taken. Kindled, implanted and unoperated control rats were treated together in all phases of sacrifice and biochemical analysis. Tissue samples were stored in liquid nitrogen before the subsequent biochemical estimation of tyrosine hydroxylase activity.

Tissues were homogenised with 20 mM potassium phosphate buffer, pH 7.4, so that 10  $\mu$ l buffer was used for each 1 mg tissue. Homogenates of tissue samples were assayed, in duplicates, for tyrosine hydroxylase activity according to the method of Hendry and Iversen (1971)<sup>8</sup>. Tissue and reagent blanks, in duplicate, were assayed simultaneously with tissue samples. Recovery was estimated by taking 10  $\mu$ l of [<sup>3</sup>H]-DOPA (specific activity 2.5 Ci/mmole, Radiochemical Centre, Amersham) through the assay, in duplicate, and compared to 10  $\mu$ l [<sup>3</sup>H]-DOPA counted directly after the addition of scintillant. The estimated percentage recovery of the method ranged between 49 and 52%. The counting efficiency of tritium was estimated to be 34%.

The results of assaying the enzyme activity are shown in Table I.

Tyrosine hydroxylase activity in brain areas of unoperated control rats were not significantly different from the normal levels previously reported<sup>9</sup>. An analysis of variance showed no significant difference in the enzyme activity in the amygdala of both the sham-operated and unoperated control rats. However, the data showed a consistent and a significant decrease in tyrosine hydroxylase activity in the left stimulated, but not the contralateral, amygdala of all kindled rats. This reduction in enzyme activity was significant at the level of  $P < 0.002$  (Mann-Whitney U-test,  $U = 3$ ) when compared to that of the contralateral amygdala. However, no correlation was found between the reduced enzyme activity and the rate of seizure development or the duration of the evoked afterdischarge.

No significant difference was found in tyrosine hydroxylase activity in the hippocampus, thalamus, hypothalamus, frontal cortex and striatum on the right and left sides of both implanted and unoperated control rats. Similarly, no significant difference in the enzyme activity was found in the hippocampus, thalamus, frontal cortex on both sides of the brain in kindled rats. However, the enzyme activity tended to be reduced in the hypothalamus and the striatum in kindled rats, but this reduction was not statistically significant.

The fact that tyrosine hydroxylase activity is significantly reduced at the site of stimulation might suggest that a persistent reduction in catecholamine production is implicated in reducing inhibition with consequent focal hyperexcitability changes in the stimulated amygdala in kindled rats. It has already been shown that kindling-induced hypersensitivity may result, in part, from a persistent decrease in postsynaptic inhibitory influences<sup>3</sup>. The absence of any significant change in catecholamine production in other brain regions studied might suggest that the mechanisms underlying the spread of the afterdischarge are different from those initiating the focal hyperexcitability in kindling convulsions.

Consequently, it is concluded that kindling produced a definite persistent decrement in tyrosine hydroxylase activity at the site of stimulation in the amygdala.

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